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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 39/12, 39/21, 39/23, 39/235, 39/245, 39/395, 39/42, 39/44, C12N 15/12, 15/35, 15/38, 15/49, 15/62, C07H 21/04

A1

(11) International Publication Number: **WO 97/38723**

(43) International Publication Date: 23 October 1997 (23.10.97)

(21) International Application Number: PCT/US97/06590

(22) International Filing Date: 15 April 1997 (15.04.97)

(30) Priority Data:
60/015,497 16 April 1996 (16.04.96) US

(60) Parent Application or Grant
(63) Related by Continuation
US 60/015,497 (CIP)
Filed on 16 April 1996 (16.04.96)

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: TARGETED VIRAL VECTORS

(57) Abstract

Viral vectors are targeted to selected cell types by blocking the wild-type viral cell binding site and incorporating a targeting agent into the vector particle. The targeting agent binds to the selected cell type by binding a molecule on the surface of the cell, or by binding a second targeting agent which binds the selected cell. Parvovirus, retrovirus, Herpes virus and Ad virus based vectors are provided. Libraries of viral vectors having the targeting agent are provided. Methods of selecting recombinant viral vectors from the libraries are also provided. Polypeptide ligands isolated from libraries of phage or viral vectors are provided.

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TARGETED VIRAL VECTORS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Provisional Patent Application USSN 60/015,497 entitled "TARGETED VIRAL VECTORS" with inventors Gang Yu, Michael Mamounas, Qicheng Yang, Jack Barber and Mang Yu, filed April 16, 1996, Attorney Docket Number 16556-0009-0. This provisional application is incorporated by reference in its entirety for all purposes. An application substantially identical to the present application was co-filed in the United States Patent Office on April 15, 1997; Attorney Docket Number 16556-0009-10; Inventors: Michael Mamounas, Gang Yu, Qicheng Yang, Qi-Xiang Li, Jack Barber and Mang Yu. This U.S. Application is incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

Cells can be stably transduced with a number of viral vectors including those derived from retroviruses, pox viruses, adenoviruses (Ads), herpes viruses and parvoviruses. Common viral vectors include those derived from murine leukemia viruses (MuLV), gibbon ape leukemia viruses (GaLV), human immuno deficiency viruses (HIV), adenoviruses, adeno associated viruses, Epstein Barr viruses, canarypox viruses, cowpox viruses, and vaccinia viruses. Viral vectors based upon retroviruses, adeno-associated viruses, herpes viruses and adenoviruses are all used as gene therapy vectors for the introduction of therapeutic nucleic acids into the cells of an organism by *ex vivo* and *in vivo* methods.

Prior art viral vectors suffer from a common problem. The vectors are typically able to transduce only those cells infected by a wild-type virus corresponding to the vector. Thus, vectors are often either unable to transfect a desired target cell, or promiscuously transfect many cell types other than a specific target cell. Thus, prior art gene therapy vectors may be ineffective, or have undesirable side effects due to transduction of non-target cells.

Prior art attempts to make viral vectors cell-specific have been only partially successful, and have been largely limited to Retroviral vectors. For instance, retroviruses are typically "pseudotyped" to alter the specificity of the virus. In this procedure, viral particle components (typically envelope surface glycoprotein

genes) homologous to a given retrovirus are transfected into a cell which produces Retroviral particles corresponding to the given retrovirus. The homologous components are expressed on the outer membrane of the virus, giving the virus an altered specificity. For instance, the vesicular stomatitis virus (VSV) *env* protein is relatively promiscuous, and can be used to expand the range of some retroviral viral vectors. However, this pseudotyping procedure suffers from two clear limitations: (1) the pseudotyped virus still infects cells within the host range of the vector because the surface of the vector includes both vector and homologous components, and (2) the homologous components are typically limited in the cells which they can infect.

In addition to pseudotyping procedures, various antibody mediated procedures have been used to alter the infectivity of particular retroviruses. *See*, Etienne-Julan (1992) *Journal of General Virology* 73: 3251-3255, and Roux *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 96: 9079-9083. However, these procedures have resulted in low viral infectivity, and result in vectors which retain the native specificity of the retrovirus.

Retroviral vectors have recently been engineered to express various cell receptor ligands in *env*, enabling the vectors to be targeted to cells which express the receptors. *See*, Cosset *et al.* (1995) *Journal of Virology* 69: 6314-6322, and Somia *et al.* (1995) *Proc. Natl. Acad. Sci. USA.* 92: 7570-7574. However, this procedure results in low infectivity of the vector, and it is not clear what the resulting host range of the engineered vectors is. In addition, described retroviral vectors only infect dividing cells.

Furthermore, the pseudotyping procedures, antibody mediated procedures and *env* engineering procedures have no clear correlate with non-enveloped viral vectors, because the structural constraints on the surface of capsid viruses is more stringent than that observed for *env* on lipid enveloped viruses. The present invention provides new strategies for viral vector targeting, solving these and other problems.

SUMMARY OF THE INVENTION

The invention provides targeted viral vectors which transfect selected cell types, and which do not enter cells through the mechanisms which ordinarily permit entry of the corresponding virus into the cell. The vectors of the invention have a nucleic acid, and a viral particle which has a targeting ligand. The particle substantially lacks a functional wild-type viral cell binding site. The Targeting ligand binds to a protein expressed on the surface of a target cell, targeting the vector to the target cell.

Typically, the wild-type viral cell binding site on the particle is deleted by deleting nucleic acid sequence which encode the site (or sites) on a virus corresponding to the vector which interact with the viral cell binding site. In one class of embodiments, the deleted site is replaced with a targeting ligand such as streptavidin, polylysine, a cell receptor ligand, an antibody against a cell receptor or an antibody binding ligand. This yields a targeted viral vector with a ligand cloned into the wild-type cell binding site. However, other arrangements are also appropriate. In cases where the wild-type site is deleted, the targeting ligand is optionally cloned into a site other than site of the deletion. In one embodiment, the wild-type site is not deleted entirely from the vectors, but is blocked, *e.g.*, by binding an antibody to the wild-type site. In this embodiment, the targeting ligand is optionally cloned into the vector, or incorporated into the monoclonal antibody which is bound to the vector(*e.g.*, by chemical modification of the antibody with a biotin label).

The nature of the viral particle varies depending on the type of vector. For example, in vectors derived from capsid viruses, such as Ad and parvoviruses, the particle is essentially a capsid. In enveloped vectors, the viral particle includes the lipid envelope and internal nucleic acid packaging components. Preferred vectors include recombinant herpes virus vectors, retroviral vectors, parvovirus vectors, Ad vectors, pox virus vectors and other vectors known to persons of skill. Typically, the vectors comprise an expression cassette for expression of a nucleic acid, which optionally codes for a protein.

The vectors include nucleic acids. In one embodiment, the nucleic acids encode part or all of the vector particle. For instance, the nucleic acid optionally encodes AAV or Ad ITRs, or retroviral LTRs, and structural proteins such as capsid proteins, envelope proteins, or the like. In other embodiments, the

nucleic acids in the vectors do not code for the vector, but are packaged by the vector. For instance, the nucleic acids optionally include a viral packaging site such that when the nucleic acid is bound by viral packaging components it is packaged into particles. For example, the nucleic acid is optionally grown in a packaging cell which expresses viral packaging components. In other embodiments, the nucleic acids are unrelated to the vector particle, and are simply associated with the particle, *e.g.*, by chemically coupling the particle to the nucleic acid.

The vectors of the invention are used to transfect a target cell with a target nucleic acid. Methods of transfection include contacting the cell with the vector *in vitro* or *in vivo*. Where the vector has a streptavidin moiety on the surface of the vector, the method optionally includes the step of binding a biotinylated antibody to the vector which binds to a molecule on the surface of the cell to direct entry of the vector into the cell.

In one embodiment, the invention provides targeted Ad nucleic acids and vectors. Preferred nucleic acids have a deletion in the L5 region of the genome, thereby reducing the natural specificity of the virus. In one preferred embodiment, a targeting ligand is cloned into the L5 region. The nucleic acid optionally encodes all of the components of the viral particle, or is optionally packaged into the viral particle with particle components encoded by heterologous nucleic acids, *e.g.*, in a packaging cell.

In another preferred embodiment, the invention provides targeted AAV vectors. These vectors include a nucleic acid and a capsid which packages the nucleic acid. Typically, the capsid has reduced or deleted specificity for the AAV cellular receptor. In one preferred embodiment, the AAV vector particle has a deletion in capsid proteins. For example, deletions in Vp1 or Vp3 are preferred and typically provide capsids with reduced specificity for the AAV receptor. For example, in one embodiment, amino acids 239-244 from Vp3 are deleted. In another embodiment, Vp1 has a deletion in the proline rich region, *e.g.*, amino acids 26-34 in Vp1. Typically, the AAV vectors include a targeting ligand expressed on the surface of the capsid. Preferred expression sites include Vp1 and Vp3. Highly preferred expression sites include amino acids 26-34 of Vp1 and 239-244 of Vp3.

Optionally, the nucleic acid packaged by the AAV particle encodes the AAV particle. However, the nucleic acid optionally is packaged into the particle

in an AAV packaging system, or an AAV transfection system such as an AAV packaging cell.

As in non-AAV and Ad vector embodiments, the Ad and AAV vectors of the invention are typically capable of introducing nucleic acids into target cells. The vectors are brought into contact with the cells, and the vectors gain entry to the cell by interaction of the targeting ligand with a molecule on the surface of the cell. The ligand is optionally incorporated into the capsid, or a molecule is optionally incorporated into the capsid which binds a second molecule such as a monoclonal antibody. For example, in one preferred embodiment the cell targeting ligand is a biotinylated protein and the capsid comprises a streptavidin moiety.

In one class of embodiments, The invention provides non-enveloped vectors such as Ad and AAV based vectors. The vectors have a capsid and a nucleic acid, where the nucleic acid is typically encapsidated in the capsid. Antibodies are optionally bound to the capsid, at either the cell binding site (thus inhibiting entry of the vector into a cell through normal cellular mechanisms), or to a ligand cloned into the cell binding site. Typically, the antibody is biotinylated. The antibody either binds to a cell surface molecule, facilitating entry of the vector into cells comprising the surface molecule, or the antibody is coupled through a molecular bridge (*e.g.*, a streptavidin intermediate) to a second antibody which recognizes a cell surface molecule.

In one class of embodiments, the invention provides combinatorial methods and libraries for selecting targeting ligands, and for optimization of vectors which comprise the targeting ligands. A targetable vector library comprising a plurality of recombinant targeted viral vectors having reduced specificity for a cellular receptor as compared to a corresponding wild-type virus is provided. In the library, each of the targeted viral vectors has a vector nucleic acid encoding a viral surface protein-targeting ligand fusion protein. A preferred library is derived from AAV, in which the recombinant targeted vectors are derived from AAV and the viral surface protein is an AAV capsid (cap) protein. Preferred targets for the vectors and libraries of the invention include cell surface proteins such as CD4 and CD34.

Method of selecting a targeted vectors using the libraries of the invention are provided. Typically a targeting nucleic acid encoding a targeting peptide which binds to a target cell is provided. This targeting nucleic acid is

randomly cloned into a vector nucleic acid comprising a mutant surface protein subsequence which encodes a viral surface protein (typically, this protein has reduced affinity for a viral receptor as compared to a wild-type virus), thereby providing a library of random insertions of the targeting nucleic acid into the mutant surface protein subsequence. This library is selected for a vector that infects a target cell, thereby selecting the targeted vector. In a preferred embodiment, a phage display library is used to select the targeting ligand.

A variety of random cloning strategies are provided, including creation of random plasmid libraries followed by subcloning into a vector, random linker insertion, transposon mutagenesis and the like.

In one embodiment, the invention includes isolated viral vectors which are isolated using the library screening methods of the invention. For example, in one embodiment, a vector having a targeting ligand which binds to a cell surface protein such as a cell receptor (*e.g.*, CD34, CD4 an HIV co-receptor surface protein or the like) is provided. The vector is made by isolating a bacteriophage particle, which specifically binds to a cell expressing the surface protein, from a random phage display library, thereby providing an identified bacteriophage particle. The relevant targeting ligand is then subcloned out of the display phage (or a synthetic sequence corresponding to the targeting ligand is made), and cloned into a viral nucleic acid. The nucleic acid is expressed in a vector packaging cell, thereby making a protein comprising the targeting ligand. The protein is incorporated into the viral vector in the packaging cell. the nucleic acid optionally encodes other features of the vector particle (capsid or envelope proteins, or the like), and is optionally packaged by the vector. In one embodiment, the vector is an AAV vector.

Polypeptides which specifically bind to CD4 are provided. Preferred polypeptides include GAVQPRGATSKLYLLRMTDK, MGEKLHRVHIRTNTPSVYSR, LEPRVAQRGQMVKFTYMRLP, HAWWKPWGWSIEALAPTAGP, and, conservative modifications thereof. Nucleic acids encoding the polypeptides are also provided. In one embodiment, the nucleic acids are encoded in a viral particle which expresses the polypeptides on the surface of the particle. For example, the polypeptides can be incorporated into a viral surface protein such as an AAV capsid protein.

BRIEF DISCUSSION OF THE DRAWING

Fig. 1. Is a schematic phage particle with a randomized 20-mer at the n-terminus of the PIII region of the phage capsid protein.

Fig. 2. Is a schematic procedure for selection of high affinity binding phage.

Fig. 3. describes theoretical mechanisms of AAV entry into a target cell, including a) AAV vector binds to target cell receptor; b) receptor-mediated entry into specific cellular compartment where c) modification/uncoating of the vector occurs; d) release from the compartment of modified vector followed by trafficking to the nucleus; e) hypothetical ligand binds to receptor that f) is internalized by mechanism not compatible with AAV transduction. Also, theoretical entry of AAV into target cell where I) receptor binding is II) independent from mechanism of internalization.

Fig. 4. is a schematic representation for construction of targetable vector library and subsequent enrichment steps.

Fig. 5. is a simplified diagram of the transposable element AT-2. Shown are 4 nucleotide U3 elements necessary for integration; unique restriction sites RE with ~15 nucleotide flanking sequences; selection marker dihydrofolate reductase gene (DHFR).

Fig. 6 shows AAV binding mutants.

DEFINITIONS

An "AAV helper virus" is a virus which supplies some or all of the functions necessary for AAV (or rAAV vector) replication which are not encoded by a wild-type AAV. Typically these functions are supplied in *trans* by viruses such as adenovirus or herpes virus during viral replication. Adenovirus and herpes virus are examples of AAV helper viruses.

An "AAV ITR sequence" refers to the sequences which comprise the palindromic terminal repeats at the 3' and 5' ends of the AAV genome. Typically, the repeats are about 150 nucleotides in length. The AAV ITR regions provide sequences for packaging the AAV provirus (*i.e.*, the AAV genome) into the AAV viral capsid. The ITR regions also form secondary structures which act as self-primers for AAV replication. Samulski (*supra*) describes AAV ITR sequences and structures.

An "adenovirus ITR" refers to the 3' and 5' terminal regions of the adenovirus genome. *See, e.g.*, Gingeras *et al.* (1982) *J. Biol. Chem.* 257:13475-13491.

An "antibody" is a polypeptide substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof which specifically bind and recognize an analyte (antigen). Recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. An exemplary immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 Kd) and one "heavy" chain (about 50-70 Kd). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (VL) and variable heavy chain (VH) refer to these light and heavy chains respectively. Antibodies exist e.g., as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce F(ab)'2, a dimer of Fab which itself is a light chain joined to VH-CH1 by a disulfide bond. The F(ab)'2 may be reduced under mild reducing conditions to break the disulfide linkage in the hinge region, thereby converting the F(ab)'2 dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (*see, Fundamental Immunology, Third Edition*, W.E. Paul, ed., Raven Press, N.Y. (1993) for a more detailed description of other antibody fragments). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such Fab' fragments may be synthesized de novo either chemically or by utilizing recombinant DNA methodology. Single chain antibodies are commonly referred to a ScAbs. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies or those synthesized de novo using recombinant DNA methodologies. An antibody or other protein specifically binds to a cognate molecule when it binds with an affinity (K_d) of at least about 10^{-6}, preferably about 10^{-8}, and usually about 10^{-9} or better.

A "cell surface receptor" or a "cell membrane receptor" is a cellular molecule expressed on the cell's outer membrane surface which is bound by a ligand. Typically, in the context of the present invention, the binding of a cell surface receptor to its cognate ligand causes endocytosis of the receptor and ligand. A "cell surface protein" is a protein expressed at least partially on the outer membrane of a cell, including transmembrane proteins and proteins associated with the outer cell membrane.

Two vectors are "complementary" when the two together encode functions necessary for vector packaging, and when each individually does not encode all of the functions necessary for packaging. Thus, for instance, when the two vectors transduce a single cell they together encode the information for production of vector particles. The use of such complementary vectors is preferred because it increases the safety of any packaging cell made by transformation with a vector.

"Encapsidation" refers to the general process of incorporating a viral genome into a viral capsid.

"Endocytosis" refers generally to the phenomenon of a cell ingesting material, *e.g.*, by phagocytosis or pinocytosis. Receptor-mediated endocytosis provides an efficient means of causing a cell to ingest material which binds to a cell surface receptor. *See*, Wu and Wu (1987) *J. Biol. Chem.* 262:4429-4432; Wagner *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:3410-3414, and EP-A1 0388 758.

The term "heterologous" when used with reference to a nucleic acid indicates that the nucleic acid comprises two or more subsequences which are not found in the same relationship to each other in nature. For instance, the nucleic acid is typically recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid. For example, in one embodiment, the nucleic acid has a promoter from one gene arranged to direct the expression of a coding sequence from a different gene. Thus, with reference to the coding sequence, the promoter is heterologous.

A "human cell" is any cell which is substantially human in origin, including organismal cells, tissue culture cells, and chimeric cells with human chromosomes.

The term "nucleic acid" refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless

otherwise limited, encompasses known analogues of natural nucleotides that hybridize to nucleic acids in manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid sequence includes the complementary sequence thereof.

A "nucleic acid vector" is a composition which can transduce, transform, transfect or infect a cell, thereby causing the cell to replicate and/or express nucleic acids and/or proteins other than those native to the cell, or in a manner not native to the cell. A vector includes a "vector nucleic acid" (ordinarily RNA or DNA) to be expressed or replicated by the cell. A vector nucleic acid optionally encodes materials to aid in achieving entry of the nucleic acid into the cell, such as a viral particle, liposome, protein coating or the like. A "cell transformation vector" is a vector which encodes a nucleic acid capable of transforming a cell once the nucleic acid is transduced into the cell.

The term "operably linked" refers to functional linkage between a nucleic acid expression control sequence (such as a promoter, or array of transcription factor binding sites) and a second nucleic acid sequence, wherein the expression control sequence directs transcription of the nucleic acid corresponding to the second sequence.

The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers.

A "potential targeting peptide" is a peptide to be tested for the its ability to target a vector to a target cell.

A "promoter" is an array of nucleic acid control sequences which direct transcription of a nucleic acid. As used herein, a promoter includes necessary nucleic acid sequences near the start site of transcription, such as, in the case of a polymerase II type promoter, a TATA element. The promoter also includes distal enhancer or repressor elements which can be located as much as several thousand base pairs from the start site of transcription. A "constitutive" promoter is a promoter which is active under most environmental conditions and states of development or cell differentiation. An "inducible" promoter responds to an extracellular stimulus.

A "receptor-binding ligand" is a biological molecule which binds to a receptor molecule on the surface of a cell. The molecule is either naturally occurring or artificial (*e.g.*, synthetic). Typically, in the context of the present invention, the binding of the receptor binding ligand to its cognate receptor results in endocytosis of the receptor binding ligand, along with materials which are attached to the receptor binding ligand.

The term "recombinant" when used with reference to a viral vector indicates that the vector comprises or is encoded by one or more nucleic acids which are derived from a nucleic acid which was artificially constructed. For example, the vector can comprise or be encoded by a cloned nucleic acid formed by joining heterologous nucleic acids as taught, *e.g.*, in Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology* volume 152 Academic Press, Inc., San Diego, CA (Berger) and in Sambrook *et al.* (1989) *Molecular Cloning - A Laboratory Manual* (2nd ed.) Vol. 1-3 (Sambrook).

A "recombinant expression cassette" is a nucleic acid construct, generated recombinantly or synthetically, with a series of specified nucleic acid elements which permit transcription of a target nucleic acid. The recombinant expression cassette can be derived from a plasmid, virus, chromosome or nucleic acid fragment. Typically, the recombinant expression cassette includes a nucleic acid to be transcribed (a target nucleic acid), and a promoter. In some embodiments, the expression cassette also includes, *e.g.*, an origin of replication, and/or chromosome integration elements such as viral LTRs or viral ITRs.

A vector has "reduced specificity" for a cellular receptor when the vector binds to the cellular receptor with lower specificity than a corresponding wild-type virus, or a vector with a wild-type viral cell recognition protein. For instance, in the examples herein, competition binding assays are used to establish that the mutant AAVs described bind to cells with less specificity than wild-type AAV. A "corresponding wild-type virus" is the virus from which the cell recognition protein on the viral vector particle is derived (*e.g.*, envelope or capsid proteins, depending on the vector). A viral "cell recognition protein" is the protein on the surface of the viral particle derived from a wild-type virus which binds a cellular receptor, thereby mediating entry of the wild-type virus. Some of these proteins, such as those derived from retroviral vectors, are glycoproteins. Typically, the vector with

reduced specificity binds to the cellular receptor at a rate which is 50% or lower than that of a vector or virus with a wild-type cell recognition protein. Usually, the vector with reduced specificity binds to the cellular receptor at a rate 25% or lower, and often 1% or lower than a vector or virus with a wild-type cell recognition protein. Binding to the cellular receptor can be measured by cellular uptake of nucleic acids, by ELISA assays, by viral competition assays, or by directly measuring the binding of the cellular receptor to the virus by labeling the receptor or the vector.

Where the viral vector with reduced specificity for the cellular receptor also includes a targeting ligand, the vector typically binds to a molecule on a cell through the targeting ligand with high specificity. Thus, specificity measurements of the binding of the viral vector to a cellular receptor other than one which binds the targeting ligand should be carried out on cells which do not comprise a molecule which binds the targeting ligand. Alternatively, the binding mediated through the targeting ligand can be independently estimated and subtracted away from the binding results.

"Sequences necessary for AAV packaging" in the context of an AAV helper nucleic acid include AAV sequences active in *trans* found between or within the AAV ITR regions which encode nucleic acids and proteins necessary for encapsidation of the rAAV nucleic acid into an AAV capsid, *e.g.*, typically the AAV capsid proteins (Vp 1, Vp 2, Vp3) and replicase proteins (Rep 78, Rep 68, Rep 40, Rep 52). In the context of the rAAV nucleic acid, "Sequences necessary for AAV packaging" refer to *cis*-active sequences which permit the rAAV nucleic acid to be "encapsidated" (packaged into an AAV viral capsid; "encapsulated" is equivalent terminology herein). These sequences typically include the AAV ITR regions.

A "Subsequence" in the context of a nucleic acid or polypeptide refers to a sequence corresponding to a portion of the nucleic acid or polypeptide. The portion is up to 100% of the nucleic acid or polypeptide, or can be only a small portion of a molecule.

A "target nucleic acid" is a nucleic acid to be transduced into a cell. The target nucleic acid can integrate into a cell's genome, or remain episomal.

The term "targeted" when used with reference to a viral vector indicates that the vector is bound by a specific subset of cells, enabling the vector to transfer associated nucleic acids into a cell of the subset.

A "targeting ligand" is a molecule which either specifically binds a cell or is bound by a second molecule or complex of molecules which specifically bind a cell. The strength or affinity of binding interactions can be expressed in terms of the dissociation constant (K_D) of the interaction, wherein a smaller K_D represents a greater affinity. Immunological binding properties of selected polypeptides can be quantified using methods well known in the art. One such method entails measuring the rates of antigen-binding site/antigen complex formation and dissociation, wherein those rates depend on the concentrations of the complex partners, the affinity of the interaction, and on geometric parameters that equally influence the rate in both directions. Thus, both the "on rate constant" (K_{on}) and the "off rate constant" (K_{off}) can be determined by calculation of the concentrations and the actual rates of association and dissociation. The ratio of K_{off}/K_{on} enables cancellation of all parameters not related to affinity and is thus equal to the dissociation constant K_D. A cell is specifically bound when the ligand (or ligand complex) has a K_D of 10^{-5} or better, preferably 10^{-6} or better, more preferably 10^{-7} or better, generally 10^{-8} or better and usually 10^{-9} or better. An alternative measure of specificity where the targeting ligand is incorporated into a viral vector is that nucleic acid associated with the vector is transferred into the cell upon incubation of the cell with the vector, while the nucleic acid is transferred into cells which lack a receptor for the targeting ligand at a substantially reduced level. Typically, vector nucleic acid is transferred into the cell with a receptor which binds the targeting ligand at a rate of at least about 5x and preferably at least about 10x that observed for a cell which lacks the receptor. A cell lacks a receptor when the cell cannot be isolated from a population of cells by FACS using an antibody against the receptor as a marker, or when an isotype matched antibody control binds to the cell with the same intensity (± about 5%) as an antibody specific for the receptor. Uptake of nucleic acid into the cell can be measured by Southern or northern analysis, quantitative PCR, by expression of encoded proteins (*e.g.*, by ELISA or western blotting) or the like.

A Targetable vector library" is a collection of related vectors, where at least one of the vectors in the collection specifically binds to a protein expressed on the surface of the cell.

A "targeted vector" is a viral vector which includes a targeting ligand. Preferably, the vector has reduced binding to a cellular receptor for the

corresponding virus. A targeting nucleic acid encodes a targeting ligand such as a peptide which binds to a protein expressed on the outer membrane of a cell.

A "target cell" is a cell to be transduced by a selected vector.

"Titers" are numerical measures of the "concentration" of a virus or viral vector compared to a reference sample, where the concentration is determined either by the activity of the virus, or by measuring the number of viruses in a unit volume of buffer. The titer of viral stocks are determined, *e.g.*, by measuring the infectivity of a solution or solutions (typically serial dilutions) of the viruses, *e.g.*, on HeLa cells using the soft agar method (*see*, Graham & Van Der eb (1973) *Virology* 52:456-467) or by monitoring resistance conferred to cells, *e.g.*, G418 resistance encoded by the virus or vector, or by quantitating the viruses by UV spectrophotometry (*see*, Chardonnet & Dales (1970) *Virology* 40:462-477).

A cell is "transduced" with a selected nucleic acid when the nucleic acid is translocated from the extracellular environment into the cell. A cell is "stably transduced" with a selected nucleic acid when the selected nucleic acid is replicated and passed on to progeny cells. A virus or vector "transduces" a cell when it transfers a nucleic acid associated with the vector into the cell. A cell is "transformed" by a nucleic acid when a nucleic acid transduced into the cell becomes stably replicated by the cell, either by incorporation of the nucleic acid into the cellular genome, or by episomal replication. A virus or vector is "infective" when it transduces a cell, replicates, and (without the benefit of any complementary virus or vector) spreads progeny vectors or viruses of the same type as the original transducing virus or vector to other cells in an organism or cell culture, wherein the progeny vectors or viruses have the same ability to reproduce and spread throughout the organism or cell culture. "Transduction" refers to the process in which a foreign nucleic acid (which is packaged in a viral particle or associated with a viral particle when outside of the cell) is introduced into a cell. The foreign nucleic acid is typically integrated into the cellular genome, but in some embodiments remains episomal. "Transfection" refers broadly to the process of causing a nucleic acid to enter a cell. In the art, the term is sometimes used to refer to a process wherein the nucleic acid is "naked," *i.e.*, not in a viral capsid or associated with other biologicals. For purposes of the present invention "transfection" refers to the process of causing a nucleic acid to enter the cell as a "naked" nucleic acid, and/or

to the process of causing a nucleic acid which is associated with other biologicals, such as nucleic acid binding molecules, viral components, receptor ligands *etc.* to enter a cell.

A "viral particle" in the context of a viral vector refers to virally derived vector material (proteins, glycoproteins, lipids, *etc.*) which package or is associated with a nucleic acid competent to be packaged by a wild-type virus in the vector. For instance, a viral particle can be a capsid (*e.g.*, where the virus is Ad or AAV), or a capsid with a lipid envelope (*e.g.*, where the virus is a retrovirus). A nucleic acid competent to be packaged by a wild-type viral particle includes packaging sequences recognized by the corresponding virus, such as the psi site in a retrovirus or the Ad or AAV ITRs.

It is expected that a viral vector viral particle will ordinarily package nucleic acid. One preferred measure of packaging is nuclease resistance of the packaged nucleic acid. If the viral vector particle protects the vector nucleic acid from a nuclease which ordinarily degrades the nucleic acid, it packages the nucleic acid. A particle protects a nucleic acid from degradation when the rate of cleavage of the packaged nucleic acid upon exposure to a nuclease is slowed by at least about 5%, and preferably at least about 10%, typically at least about 20%, more typically at least about 30%, usually at least about 40%, ordinarily at least about 50%, and generally at least about 60%. In preferred embodiments, cleavage by the nuclease is slowed by 90% or more.

A "viral vector" is a nucleic acid vector which has components derived from a virus which aid in packaging the nucleic acid and/or transferring the nucleic acid into a cell. For example, an AAV viral vector has AAV components such as AAV capsid proteins, and an HIV viral vector has HIV components such as HIV *gag* and *env* proteins. Viral vectors are optionally heterologous, *i.e.*, they optionally comprise components from more than one virus. For example, a vector optionally comprises homologous components from more than one virus (*e.g.*, *env* proteins from MuLV and VSV on the surface of a viral particle), or complementary proteins from more than one virus (*e.g.*, VSV *env* proteins on the surface of a retroviral particle with MuLV capsid antigen proteins packaging a nucleic acid inside of the retroviral outer lipid envelope).

A "viral surface protein" is a protein expressed on the outer surface of the protein, such as a capsid protein or an envelope protein. A "viral surface protein-targeting ligand fusion protein" is a fusion protein which has a surface protein domain, which optionally encodes part or all of the surface protein, linked to a ligand protein domain. Ordinarily, the fusion protein is produced by recombinantly joining a nucleic acid which encodes a targeting ligand peptide to a nucleic acid which encodes at least a portion of the surface protein, and expressing the resulting recombinant molecule.

A "wild-type viral cell binding site" is an epitope on the surface of a viral vector which is derived from an epitope on the surface of a wild type virus which mediates entry of the virus into a cell within the host range of the virus. A "wild-type cell viral binding site" is the site on the cell bound by the epitope on the wild-type virus (*i.e.*, the cellular receptor for the virus).

A functional AAV receptor" is a cell surface protein which is bound by a wild-type AAV, wherein binding of the cell surface protein facilitates entry of the AAV into the cell, *e.g.*, by receptor mediated endocytosis.

DETAILED DISCUSSION OF THE INVENTION AND PREFERRED EMBODIMENTS

Several cell transformation vectors are becoming increasingly useful as gene therapy vectors. Some of the most widely used cell transformation vectors include those derived from wild-type viruses. Typically, the vectors have viral nucleic acid subsequences which permit packaging of the nucleic acid into a viral particle corresponding to the virus from which the sequence is derived. For instance, retroviruses have a packaging site which is typically located adjacent to the 5' LTR, *e.g.*, next to the gene for *gag*. *See*, Aldovani *et al.* (1990) *Journal of Virology* 64(5): 1920-1926. The packaging sites for parvoviruses such as B 19 and AAV are located in the viral ITRs. *See*, Samulski, *supra*. Adenovirus packaging sites are located in the adenoviral ITRs.

Many vectors also include sequences for chromosomal integration of vector nucleic acids into a host chromosome. Such sequences include parvovirus (particularly AAV) ITRs, retroviral LTRs and various known transposable elements. Transcription cassettes competent to express genes of interest in a cell are often

placed between these chromosome integration elements for integration into the host chromosome.

Targeting vectors to particular cell types is of interest for both *in vitro* and *in vivo* transformation of cells. For instance, in replacement gene therapy where defective or absent genes are replaced using cell transformation vectors, only a subset of all an organism's cells ideally express a replacement gene. Similarly, only selected cell types are targets for intracellular immunization procedures which immunize cells against virus infection by expressing anti-viral agents in the cells. When using gene therapeutic procedures against cancers, it is desirable to limit expression of anti-cancer agents to cancer cells.

The present invention provides a general approach to making targeted gene therapy vectors. The first step in making targeted vectors of the invention is to destroy the natural specificity of a given vector. This is done to prevent the vector from infecting cells within the host range of the virus from which the vector is derived. The natural specificity of a vector is destroyed by recombinantly deleting or mutating the site on the vector which is ordinarily recognized by a cellular receptor during entry of the vector (the viral cell binding site). For instance, where the vector is derived from a retrovirus, the envelope glycoproteins are mutated to prevent recognition by a cellular receptor. For instance, where the cellular receptor is CD4 (*i.e.*, when the vector has a particle based upon a primate lentivirus), the envelope glycoproteins are mutated such that $CD4^+$ cells no longer permit entry of the vector.

Similarly, where the vector is derived from a capsid-based virus such as an Ad or AAV, the capsid proteins are recombinantly altered so that cells infectable by the corresponding virus no longer permit entry of the modified vector.

As an alternative to recombinant modification, the viral cell binding site can be modified chemically or by binding an antibody (or other moiety) to the site to prevent interaction of the site with a cell.

The second step in making a targeted vector is to incorporate a targeting molecule into the vector. The targeting molecule is typically a peptide epitope recognized by a cell, or is a peptide moiety recognized by a coupling complex. For instance, the epitope can be the binding site for a cellular receptor (*e.g.*, IL-2, IL-4, FcγI, FcγII and FcγIII receptors, CD4, CD8, CD34, HIV co-

receptors such as a member of the 7 transmembrane receptor family, such as fusin, CKR5, CKR-3 or CKR-2b or other chemokine receptors) on the surface of a cell. Many cellular receptor-ligand interactions are known, including those described in Hulme (ed) *Receptor Ligand Interactions* (1992) Oxford University Press (Hulme 1) and the references therein, and Hulme (ed) *Receptor Effector Coupling* (1990) Oxford University Press (Hulme 2) and the references therein. Methods of measuring binding to cellular receptors are also known, and described in Hulme 1 and Hulme 2.

Recently, a seven-transmembrane domain protein was shown to serve as an accessory factor for T-cell-tropic (T-tropic) HIV-1 isolates. *See,* Feng *et al.*, *Science* 272:872-877, 1996, and Berson *et al.* (1996) *J Virol* 70 (9): 6288-95. Expression of this glycoprotein, termed fusin, in murine, feline, simian and quail cell lines, in conjunction with human CD4, rendered these cells permissive for HIV-1 envelope glycoprotein (Env)-mediated infection. Fusin is an appropriate target which a targeted vector of the invention can be directed against.

A second member of the seven-transmembrane domain protein family, the beta-chemokine receptor CKR-5 (alternately known as "CC-CKR5" or as "CCR-5"), mediates infection of macrophage by M-tropic HIV viruses. Co-expression of CKR-5 with CD4 enables nonpermissive cells to form syncytia with cells infected by M-tropic, but not T-tropic, HIV-1 env proteins. Expression of CKR-5 and CD4 permits entry of M-tropic, but not T-tropic, virus strain. *See,* Doranz *et al.* (1996) *Cell* 85 (7): 1149-58; Feng *et al.* (1996) *Science* 272 (5263): 872-7; Alkhatib *et al.* (1996) *Science* 272 (5270): 1955-8, and Deng *et al.* (1996) *Nature* 381 (6584): 661-6. Some T cells also express CKR-5 (*e.g.*, in addition to fusin), and CKR-5 can also mediate infection of M-tropic HIV viruses into these T cells. CKR-5 is an appropriate target which a targeted vector of the invention can be directed against. Sequence for the CKR5 receptor is found in GenBank at Accesion U57840. *See also,* Combadiere *et al.* (1996) *J. Leukoc. Biol.* 60 (1), 147-152 and Combadiere May 9, 1996 *Direct Submission to GenBank*).

A dual-tropic primary HIV-1 isolate (89.6) utilizes both Fusin and CKR-5 as entry cofactors. *See,* Doranz *et al.*, *id.* Cells expressing the 89.6 env protein form syncytia with QT6 cells expressing CD4 and either Fusin or CKR-5. The beta-chemokine receptors CKR-3 and CKR-2b support HIV-1 89.6 env-mediated

syncytia formation. These known chemokine receptors are also appropriate targets for the targeted vectors of the invention.

Combinations of cell surface molecules are also appropriate targets. For example, a targeting ligand optionally binds more than one cell surface protein, such as CD4 and an HIV co-receptor molecule such as fusin or CKR5.

In another preferred embodiment, the targeting molecule is streptavidin, and the vector is targeted to a cell by binding a second biotinylated targeting molecule to the streptavidin through the biotinylation site. This second targeting molecule can be an antibody which recognizes a particular cell type, or a cell receptor ligand for a receptor found on a target cell type.

Making Vector Nucleic Acids

The vectors of the invention comprise recombinant nucleic acids which optionally encode targeting molecules, expression cassettes, viral packaging sites, chromosomal integration elements (*e.g.*, AAV ITRs or retroviral LTRs) and the like. Given the strategy for making the vectors of the present invention, one of skill can construct a variety of vectors containing functionally equivalent nucleic acids. Cloning methodologies to accomplish these ends, and sequencing methods to verify the sequence of nucleic acids are well known in the art. Examples of appropriate cloning and sequencing techniques, and instructions sufficient to direct persons of skill through many cloning exercises are found in Berger and Kimmel, *Guide to Molecular Cloning Techniques, Methods in Enzymology* volume 152 Academic Press, Inc., San Diego, CA (Berger); Sambrook *et al.* (1989) *Molecular Cloning - A Laboratory Manual* (2nd ed.) Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY, (Sambrook); and *Current Protocols in Molecular Biology*, F.M. Ausubel *et al.*, eds., Current Protocols, a joint venture between Greene Publishing Associates, Inc. and John Wiley & Sons, Inc., (1994 Supplement) (Ausubel). Product information from manufacturers of biological reagents and experimental equipment also provide information useful in known biological methods. Such manufacturers include the SIGMA chemical company (Saint Louis, MO), R&D systems (Minneapolis, MN), Pharmacia LKB Biotechnology (Piscataway, NJ), CLONTECH Laboratories, Inc. (Palo Alto, CA), Chem Genes Corp., Aldrich Chemical Company (Milwaukee, WI), Glen Research, Inc., GIBCO BRL Life Technologies, Inc. (Gaithersberg, MD), Fluka Chemica-Biochemika

Analytika (Fluka Chemie AG, Buchs, Switzerland), Invitrogen, San Diego, CA, and Applied Biosystems (Foster City, CA), as well as many other commercial sources known to one of skill.

In one preferred embodiment, the steps of reducing the natural specificity of a vector and adding a targeted specificity are combined. In this embodiment, a nucleic acid encoding targeting peptide such as a cell receptor ligand, or a streptavidin peptide is used in an insertional mutagenesis procedure. The nucleic acid is inserted into the coding region for a capsid or surface envelope protein at locations which potentially encode the natural viral cell binding site on the surface of the corresponding wild-type vector. The insertion is performed such that the reading frame of the coding region is not altered. Resulting vectors are then screened for their ability to enter a target cell through the targeting peptide, and for their inability to enter a cell through the receptor which recognizes a corresponding virus. An example of insertional mutagenesis on herpes virus is found in Chiang *et al.* (1994) 68(4): 2529-2543.

The nucleic acid compositions of this invention, whether RNA, cDNA, genomic DNA, or a hybrid of the various combinations, are isolated from biological sources or synthesized *in vitro*. The nucleic acids of the invention are present in transformed or transfected whole cells, in transformed or transfected cell lysates, or in a partially purified or substantially pure form.

In vitro amplification techniques suitable for generating and amplifying sequences for use as molecular probes or generating nucleic acid fragments for subsequent subcloning are known. Examples of techniques sufficient to direct persons of skill through such *in vitro* amplification methods, including the polymerase chain reaction (PCR) the ligase chain reaction (LCR), Qβ-replicase amplification and other RNA polymerase mediated techniques (*e.g.*, NASBA) are found in Berger, Sambrook, and Ausubel, as well as Mullis *et al.*, (1987) U.S. Patent No. 4,683,202; *PCR Protocols A Guide to Methods and Applications* (Innis *et al.* eds) Academic Press Inc. San Diego, CA (1990) (Innis); Arnheim & Levinson (October 1, 1990) *C&EN* 36-47; *The Journal Of NIH Research* (1991) 3, 81-94; (Kwoh *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86, 1173; Guatelli *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87, 1874; Lomell *et al.* (1989) *J. Clin. Chem* 35, 1826; Landegren *et al.*, (1988) *Science* 241, 1077-1080; Van Brunt (1990) *Biotechnology*

8, 291-294; Wu and Wallace, (1989) *Gene* 4, 560; Barringer *et al.* (1990) *Gene* 89, 117, and Sooknanan and Malek (1995) *Biotechnology* 13: 563-564. Improved methods of cloning *in vitro* amplified nucleic acids are described in Wallace *et al.*, U.S. Pat. No. 5,426,039.

Oligonucleotides for use as probes, *e.g.*, in *in vitro* amplification methods, for use as gene probes, or as inhibitor components (*e.g.*, ribozymes) are typically synthesized chemically according to the solid phase phosphoramidite triester method described by Beaucage and Caruthers (1981), *Tetrahedron Letts.*, 22(20):1859-1862, *e.g.*, using an automated synthesizer, as described in Needham-VanDevanter *et al.* (1984) *Nucleic Acids Res.*, 12:6159-6168. Oligonucleotides can also be custom made and ordered from a variety of commercial sources known to persons of skill. Purification of oligonucleotides, where necessary, is typically performed by either native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson and Regnier (1983) *J. Chrom.* 255:137-149. The sequence of the synthetic oligonucleotides can be verified using the chemical degradation method of Maxam and Gilbert (1980) in Grossman and Moldave (eds.) Academic Press, New York, *Methods in Enzymology* 65:499-560.

Making Conservative Substitutions

One of skill can easily generate a nucleic acid sequence by reference to a given polypeptide sequence by reference to the genetic code, using synthetic or recombinant techniques as described, *supra*. For example, one of skill can easily make nucleic acids encoding the polypeptides GAVQPRGATSKLYLLRMTDK, MGEKLHRVHIRTNTPSVYSR, LEPRVAQRGQMVKFTYMRLP, and HAWWKPWGWSIEALAPTAGP, as well as conservative modifications of these proteins. Conservative modifications of the vectors of the invention are also appropriate.

One of skill will appreciate that many conservative variations of the nucleic acid and polypeptide constructs of the invention disclosed yield functionally identical constructs. For example, due to the degeneracy of the genetic code, "silent substitutions" (*i.e.*, substitutions of a nucleic acid sequence which do not result in an alteration in an encoded polypeptide) are an implied feature of *every* nucleic acid sequence which encodes an amino acid. Similarly, "conservative amino acid substitutions," in one or a few amino acids in an amino acid sequence of a

packaging or packageable construct are substituted with different amino acids with highly similar properties and are also readily identified as being highly similar to a disclosed construct. Such conservatively substituted variations of each explicitly disclosed sequence are a feature of the present invention.

Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given polypeptide. For instance, the codons CGU, CGC, CGA, CGG, AGA, and AGG all encode the amino acid arginine. Thus, at every position where an arginine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of "conservatively modified variations." Every nucleic acid sequence which encodes a polypeptide also describes every possible silent variation. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine) can be modified to yield a functionally identical molecule by standard techniques. Accordingly, each "silent variation" of a nucleic acid which encodes a polypeptide is implicit in any described sequence. Furthermore, one of skill will recognize that individual substitutions, deletions or additions which alter, add or delete a single amino acid or a small percentage of amino acids (typically less than 5%, more typically less than 1%) in an encoded sequence are "conservatively modified variations" where the alterations result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. The following six groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Serine (S), Threonine (T); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W). *See also*, Creighton (1984) *Proteins* W.H. Freeman and Company. In a preferred embodiment, a nucleic acid which encodes a polypeptide is optimized for translation efficiency by selecting codons which are prevalent in the cell type which the virus is to be expressed in. Species codon bias tables are well known, and in common use.

A complementary nucleic acid refers to a nucleic acid complementary to the sense strand. Thus, a sense strand encoding a given polypeptide hybridizes under stringent conditions to the complementary strand. Stringent hybridization or

wash conditions in the context of nucleic acid hybridization experiments such as Southern and northern hybridizations are sequence dependent, and are different under different environmental parameters. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology–Hybridization with Nucleic Acid Probes* part I chapter 2 "overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York. Generally, highly stringent hybridization and wash conditions are selected to be about 5° C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and ph. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Very stringent conditions are selected to be equal to the T_m for a particular probe. An example of stringent hybridization conditions for hybridization of complementary nucleic acids which have more than 100 complementary residues on a filter in a Southern or northern blot is 50% formalin with 1 mg of heparin at 42°C, with the hybridization being carried out overnight. An example of stringent wash conditions is a .2x SSC wash at 65°C for 15 minutes (*see,* Sambrook, *supra* for a description of SSC buffer). Often the high stringency wash is preceded by a low stringency wash to remove background probe signal. An example low stringency wash is 2x SSC at 40°C for 15 minutes. In general, a signal to noise ratio of 2x (or higher) than that observed for an unrelated probe in the particular hybridization assay indicates detection of a specific hybridization. A coding nucleic acid refers to the sense strand of a nucleic acid. Nucleic acids which do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, *e.g.*, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code.

One of skill will recognize many ways of generating alterations in a given nucleic acid construct. Such well-known methods include site-directed mutagenesis, PCR amplification using degenerate oligonucleotides, exposure of cells containing the nucleic acid to mutagenic agents or radiation (mutagenic agents and radiation are collectively referred to herein as "mutagens"), chemical synthesis of a desired oligonucleotide (*e.g.*, in conjunction with ligation and/or cloning to generate large nucleic acids) and other well-known techniques. *See,* Giliman and Smith

(1979) *Gene* 8:81-97, Roberts *et al.* (1987) *Nature* 328:731-734 and Sambrook, Innis, Ausbel, Berger, Needham VanDevanter and Mullis (*all supra*).

One of skill can select a desired nucleic acid of the invention based upon the sequences provided and upon knowledge in the art regarding viruses generally. The life-cycle, genomic organization, developmental regulation and associated molecular biology of viruses have been the focus of over a century of intense research. The specific effects of many mutations in viral genomes are known. Moreover, general knowledge regarding the nature of proteins and nucleic acids allows one of skill to select appropriate sequences with activity similar or equivalent to the nucleic acids and polypeptides disclosed in the sequence listings herein. Conservative amino acid substitutions are described herein.

Finally, most modifications to nucleic acids are evaluated by routine screening techniques in suitable assays for the desired characteristic. For instance, changes in the immunological character of encoded polypeptides can be detected by an appropriate immunological assay. Modifications of other properties such as nucleic acid hybridization to a complementary nucleic acid, redox or thermal stability of encoded proteins, hydrophobicity, susceptibility to proteolysis, or the tendency to aggregate are all assayed according to standard techniques.

Making Parvovirus Vectors

A general introduction to human parvoviruses is found, *e.g.*, in Pattison (1994) *Principles and Practice of Clinical Virology* (Chapter 23) Zuckerman *et al.* eds, John Wiley & Sons Ltd. and the references therein. The best characterized of the human parvoviruses are B19 and AAV, both of which are used as the basis for cell transformation vectors, *e.g.*, for gene therapy. AAVs are of particular use for the transformation of cells *in vivo* and *ex vivo* with target nucleic acids.

AAVs utilize helper viruses such as adenovirus or herpes virus to achieve productive infection. In the absence of helper virus functions, AAV integrates (site-specifically) into a host cell's genome, but the integrated AAV genome has no pathogenic effect. The integration step allows the AAV genome to remain genetically intact until the host is exposed to the appropriate environmental conditions (*e.g.*, a lytic helper virus), whereupon it re-enters the lytic life-cycle. Samulski (1993) *Current Opinion in Genetic and Development* 3:74-80 and the

references cited therein provides an overview of the AAV life cycle. For a general review of AAVs and of the adenovirus or herpes helper functions *see*, Berns and Bohensky (1987) *Advanced in Virus Research*, Academic Press., 32:243-306. The genome of AAV is described in Laughlin *et al.* (1983) *Gene*, 23:65-73. Expression of AAV is described in Beaton *et al.* (1989) *J. Virol.*, 63:4450-4454.

AAV-based vectors are used to transduce cells with target nucleic acids, *e.g.*, in the *in vitro* production of nucleic acids and peptides, and in *in vivo* and *ex vivo* gene therapy procedures. *See*, West *et al.* (1987) *Virology* 160:38-47; Carter *et al.* (1989) U.S. Patent No. 4,797,368; Carter *et al.* WO 93/24641 (1993); Kotin (1994) *Human Gene Therapy* 5:793-801; Muzyczka (1994) *J. Clin. Invst.* 94:1351 and Samulski (*supra*) for an overview of AAV vectors. Construction of recombinant AAV vectors are described in a number of publications, including Lebkowski, U.S. Pat. No. 5,173,414; Tratschin *et al.* (1985) *Mol. Cell. Biol.* 5(11):3251-3260; Tratschin, *et al.* (1984) *Mol. Cell. Biol.*, 4:2072-2081; Hermonat and Muzyczka (1984) *Proc. Natl. Acad. Sci. USA*, 81:6466-6470; McLaughlin *et al.* (1988) and Samulski *et al.* (1989) *J. Virol.*, 63:03822-3828. Cell lines that can be transformed by rAAV include those described in Lebkowski *et al.* (1988) *Mol. Cell. Biol.*, 8:3988-3996.

Recombinant AAV vectors (rAAV vectors) deliver foreign nucleic acids to a wide range of mammalian cells (Hermonat & Muzycka (1984) *Proc Natl Acad Sci USA* 81:6466-6470; Tratschin *et al.* (1985) *Mol Cell Biol* 5:3251-3260), integrate into the host chromosome (McLaughlin *et al.* (1988) *J Virol* 62: 1963-1973), and show stable expression of the transgene in cell and animal models (Flotte *et al.* (1993) *Proc Natl Acad Sci USA* 90:10613-10617). rAAV vectors are able to infect non-dividing cells (Podsakoff *et al.* (1994) *J Virol* 68:5656-66; Flotte *et al.* (1994) *Am. J. Respir. Cell Mol. Biol.* 11:517-521). Further advantages of rAAV vectors include the lack of an intrinsic strong promoter, thus avoiding possible activation of downstream cellular sequences, and the vector's naked icosohedral capsid structure, which renders the vectors stable and easy to concentrate by common laboratory techniques.

rAAV vectors are used to inhibit, *e.g.*, viral infection, by including anti-viral transcription cassettes in the rAAV vector. For example, Chatterjee *et al.* (*Science* (1992), 258: 1485-1488, hereinafter Chatterjee *et al.* 1) describe anti-sense

inhibition of HIV-1 infectivity in target cells using an rAAV vector with a constitutive expression cassette expressing anti-TAR RNA. Chatterjee *et al.* (PCT application PCT/US91/03440 (1991), hereinafter Chatterjee *et al.* 2) describe rAAV vectors, including rAAV vectors which express antisense TAR sequences. Chatterjee and Wong (*Methods, A companion to Methods in Enzymology* (1993), 5: 51-59) further describe rAAV vectors for the delivery of antisense RNA.

rAAV vectors have several properties which make them preferred gene delivery systems in clinical settings. They have no known mode of pathogenesis and 80% of people in the United States are currently seropositive for AAV (Blacklow *et al.* (1971) *J Natl Cancer Inst* 40:319-327; Blacklow *et al.* (1971) *Am J Epidemiol* 94:359-366). Because rAAV vectors have little or no endogenous promoter activity, specific promoters may be used, depending on target cell type. rAAV vectors can be purified and concentrated so that multiplicities of infection exceeding 1.0 can be used in transduction experiments. This allows virtually 100% of the target cells in a culture to be transduced, eliminating the need for selection of transduced cells.

Targetable AAVs

The basic strategy for creating a targetable rAAV is to destroy the normal binding region on the AAV capsid and to add a targeting ligand to the capsid. As set forth in the examples below, the natural specificity of the AAV capsid was destroyed by mutations in either Vp1 or Vp3, while still retaining the ability of the viral capsid to protect the encapsidated nucleic acid from DNAse 1 digestion. Combinatorial methods for rapidly screening AAV mutants are also provided below.

These AAV vectors are made targetable in one of several ways. In one preferred embodiment, a nucleic acid encoding a streptavidin peptide is cloned into a capsid protein gene, resulting in a capsid protein which binds biotin. Vectors comprising the recombinant capsid protein are targeted to particular cells by binding a biotinylated targeting agent to the vector which agent is recognized by a particular type of cell. For instance, a biotinylated antibody which binds a particular cell type is used to target the vector to the cell type. In one particularly preferred embodiment, the streptavidin nucleic acid is cloned into the site of the mutation (*e.g.*, deletion) which makes the capsid unable to bind the AAV cellular receptor.

In other preferred embodiments, a targeting ligand which binds to a cell receptor, such as a single chain antibody protein against CD34, the C4 peptide which binds to CD4, or other targeting ligands are cloned into the AAV vector. In one embodiment described more fully below, a targeting ligand is identified by combinatorial screening methods from phage libraries of peptides. The targeting ligand is typically about 20 amino acids in length, although longer or shorter peptides are also useful.

Retroviral Vectors

Retrovirus-based vectors are useful due to their ability to transduce cells efficiently with target nucleic acids, and because of their ability to integrate a target nucleic acid into a cellular genome. The majority of approved gene transfer trials in the United States rely on replication-defective retroviral vectors harboring a therapeutic polynucleotide sequence as part of the retroviral genome (*see*, Miller *et al.* (1990) *Mol. Cell. Biol.* 10:4239 (1990); Kolberg (1992) *J. NIH Res.* 4:43, and Cornetta et al. *Hum. Gene Ther.* 2:215 (1991)). Widely used vectors include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), Simian Immuno deficiency virus (SIV), human immuno deficiency virus (HIV), and combinations thereof. *See*, *e.g.*, Buchscher *et al.* (1992) *J. Virol.* 66(5) 2731-2739; Johann *et al.* (1992) *J. Virol.* 66 (5):1635-1640 (1992); Sommerfelt *et al.*, (1990) *Virol.* 176:58-59; Wilson *et al.* (1989) *J. Virol.* 63:2374-2378; Miller et al., *J. Virol.* 65:2220-2224 (1991); Wong-Staal *et al.*, PCT/US94/05700; Rosenburg and Fauci (1993) in *Fundamental Immunology, Third Edition* Paul (ed) Raven Press, Ltd., New York and the references therein, and Yu *et al.*, *Gene Therapy* (1994) 1:13-26.

Various methods of targeting retroviruses have been described. Roux *et al.* (1989) *Proc. Natl Acad. Sci. USA* 96:9079-9083 describe a technique for making biotinylated antibodies and targeting retroviruses to MHC receptors using biotinylated antibodies and streptavidin. Cosset *et al.*, *supra* describe a technique for targeting retroviral vectors to cells expressing epidermal growth factor receptors (EGFR) or Ram-1.

Targetable Retroviruses of the invention

The basic strategy for creating a targetable retroviral vector is to destroy the normal binding region on the retroviral envelope and to add a targeting

ligand to the envelope. As described for rAAVs, this technique is performed recombinantly, or by adding a targeting agent to the viral particles. Because the retroviral particles have a lipid envelope, targeting molecules which bridge the lipid envelope are optionally added to the envelope, *e.g.*, using liposomes comprising the targeting agent.

One class of preferred retroviral vectors of the invention have a streptavidin peptide cloned into the N-terminal region of the relevant retroviral surface protein. For instance, MLV vectors with *Sfi* 1 and *Not* 1 restriction sites at codon 6 are available. *See*, Cosset, *id.* The retroviral vectors are targeted to a cell by complexing the retroviral particle comprising the streptavidin site with an antibody or other protein specific for a target cell type. Similarly, any other targeting ligand as described herein can be cloned into a retroviral vector in the N terminal region of an *env* gene. As described *supra*, combinatorial methods for optimizing these vectors are provided.

Herpes Virus Vectors

An introduction to herpes viruses, including herpes simplex, varicella zoster, cytomegalovirus, Epstein-bar virus and human herpes viruses 6 and 7 are found in Zuckerman *et al.* (eds) (1994) John Wiley & Sons, New York Chapter 1 (*see*, Cleator and Klapper, Kangoro and Harper, Griffiths, Crawford and Fox *et al.*). A variety of Herpes virus vectors are described in Cochran *et al.* (1993) U.S. Pat. No. 5,223,424.

Glycoprotein gD on the HSV envelope, and the homologous glycoprotein on other herpes envelopes, mediate infectivity of the virus. Chiang *et al.* (1994) *Journal of Virology* 68(4) 2529-2553 and the references therein describe the functional regions of the protein, including the receptor binding site. In the methods of the invention, the protein is disabled for its ability to bind the herpes virus receptor, either recombinantly, or by binding an antibody or other protein to the receptor. A targeting molecule is then incorporated into the viral envelope, either recombinantly into the gD protein, or using liposomes, or by binding the targeting molecule to the surface of the vector.

One class of preferred herpes virus vectors of the invention have a streptavidin peptide cloned into residues from the region from 270-310 of the gD surface protein. The herpes virus vectors are targeted to a cell by complexing the

herpesvirus particle comprising the streptavidin site with an antibody specific for a target cell type. Other preferred herpes vectors utilize any other peptide targeting ligand described herein cloned into the region from 270-310 of the gD protein.

Adenovirus Vectors

An introduction to adenoviruses is found in Zuckerman *et al.* (eds) (1994) John Wiley & Sons, New York Chapter 7 (*see*, Sharp and Wadell) and the references therein. Adenovirus vectors for gene delivery are described, *e.g.*, in Denefle *et al.* WO 95/238967; Vigne *et al.* (1995) *Restorative Neurology and Neuroscience* 8: 35-36; Kremer and Perricaudet (1995) *British Medical Bulletin* 51(1): 31-44; Haddada *et al.* (1995) in *The Molecular Repitoire of Adenoviruses III, Biology and Pathogenesis* 297-304, Springer Verlag, New York, and Randrianson-Jewtoukoff and Perricaudet (1995) *Biologicals* 23: 145-157.

Adenovirus capsids are essentially composed of three proteins: hexons, pentons, and penton fibers. Each virion capsid contains 240 hexon proteins that comprise the bulk of the virion icosahedral shell. At each vertex of the capsid is a penton base protein which is non-covalently attached to the amino terminus of the penton fiber protein. At the carboxy terminus of the penton fiber there is a knob-like structure which mediates receptor binding of the virus.

In the constructs of the invention, deletions or other mutations are made in the binding domain of the carboxy terminus of the fiber protein which is located in region L5 of the adenoviral genome (85-95 mμ). Targeting ligands are then cloned into the capsid protein genes, preferably into the mutated region of the pention fiber.

In one preferred embodiment, the coding sequence for streptavidin is cloned into the region corresponding to the fiber binding domain. Streptavidin is capable of high affinity binding to biotin (Kd $\sim 10^{-13}$) and procedures are readily available for biotinylating proteins of interest. Hence, biotinylated antibodies (or other ligands) of choice are used to form non-covalent linkage with adenovirus containing fiber/streptavidin fusion proteins. This modified vector allows for cloning genes of interest into typical adenovirus cloning regions and, upon linkage of biotinylated targeting protein, is able to specifically bind to target cells with high affinity, without significant non-target cell interactions.

Similarly, other preferred Ad vectors utilize any other peptide targeting ligand described herein cloned into the L5 region.

Making Antibodies

In many embodiments, the vectors of the invention are targeted by binding antibodies specific for a target cell to the vector, or single chain antibodies are recombinantly expressed on the surface of a vector of the invention. Methods of producing polyclonal and monoclonal antibodies are known to those of skill in the art, and many anti-cellular antibodies, including antibodies which recognize tumors, virally infected cells, hematopoietic cells and other targets for gene therapy are commercially available.

For the construction of antibodies generally, *See, e.g.*, Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; and Harlow and Lane (1989) *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY; Stites *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY; and Kohler and Milstein (1975) Nature 256: 495-497. Such techniques include antibody preparation by selection of antibodies from libraries of recombinant antibodies in phage or similar vectors. *See*, Huse *et al.* (1989) *Science* 246: 1275-1281; and Ward, *et al.* (1989) *Nature* 341: 544-546. Specific monoclonal and polyclonal antibodies and antisera will usually bind with a K_D of at least about .1 mM, more usually at least about 1 μM, preferably at least about .1 μM or better, and most typically and preferably, .01 μM or better.

As described *supra*, the vectors of the invention optionally comprise or encode a target molecule such as a protein or ribozyme. A particular protein expressed by the recombinant expression cassettes of the invention can be quantified by a variety of immunoassay methods, *i.e.*, when expression of a protein is used to monitor whether the vector transduces a target cell. For a review of immunological and immunoassay procedures in general, see Stites and Terr (eds.) 1991 *Basic and Clinical Immunology* (7th ed.). Moreover, the immunoassays of the present invention can be performed in any of several configurations, *e.g.*, those reviewed in Maggio (ed.) (1980) *Enzyme Immunoassay* CRC Press, Boca Raton, Florida; Tijan (1985) "Practice and Theory of Enzyme Immunoassays," *Laboratory Techniques in*

Biochemistry and Molecular Biology, Elsevier Science Publishers B.V., Amsterdam; Harlow and Lane, *supra*; Chan (ed.) (1987) *Immunoassay: A Practical Guide* Academic Press, Orlando, FL; Price and Newman (eds.) (1991) *Principles and Practice of Immunoassays* Stockton Press, NY; and Ngo (ed.) (1988) *Non isotopic Immunoassays* Plenum Press, NY.

Coupling Targeting Proteins To Biological Agents

In the invention, antibodies or other proteins such as cell receptor ligands are optionally used as targeting agents. These proteins are either recombinantly fused to a viral surface protein, such as an adenoviral or adeno associated viral capsid protein as described *supra*, or are added to the vector. In one embodiment, these proteins are chemically bound to a biological agent such as biotin which binds a cognate on the surface of the vector particle, *e.g.*, a streptavidin moiety. The procedure for attaching an agent to a protein will vary according to the chemical structure of the agent. Antibodies are proteins which contain a variety of functional groups; *e.g.*, carboxylic acid (COOH) or free amine ($-NH_2$) groups, which are available for reaction with a suitable functional group on an agent molecule to bind the agent thereto. Alternatively, the protein and/or agent may be derivatized to expose or attach additional reactive functional groups. The derivatization may involve attachment of any of a number of linker molecules such as those available from Pierce Chemical Company, Rockford, Illinois. A bifunctional linker having one functional group reactive with a group on a particular agent, and another group reactive with an protein, may be used to form the desired protein conjugate. Alternatively, derivatization may involve chemical treatment of the protein; *e.g.*, glycol cleavage of the sugar moiety of the glycoprotein protein with periodate to generate free aldehyde groups. The free aldehyde groups on the protein may be reacted with free amine or hydrazine groups on an agent to bind the agent thereto. (See U.S. Patent No. 4,671,958). Procedures for generation of free sulfhydryl groups on antibodies or protein fragments are also known (See U.S. Pat. No. 4,659,839). Many procedure and linker molecules for attachment of various compounds including radionuclide metal chelates, toxins and drugs to proteins such as antibodies are known. *See*, for example, European Patent Application No. 188,256; U.S. Patent Nos. 4,671,958, 4,659,839, 4,414,148, 4,699,784; 4,680,338;

4,569,789; and 4,589,071; and Borlinghaus *et al. Cancer Res.* 47: 4071-4075 (1987)).

In a preferred embodiment, biotin is coupled to the targeting protein to facilitate binding of the targeting protein to the viral particle. Biotin structure and biological activity are described, *e.g.*, in Stryer (1988) *Biochemistry*, third edition Freeman and Co. NY. Methods of biotinylating proteins are widely known and used, and biotinylating reagents and kits are widely available, *e.g.*, from Sigma, Aldrich, and Pierce. Harlow and Lane describe methods of biotinylating proteins such as antibodies in *Antibodies: A Laboratory Manual* (1988) By Cold Spring Harbor Laboratory Press. To prepare biotinylated protein, proteins are incubated with a biotinylating reagent such as NHS-LC-biotin (Pierce) overnight at 4°C, and then purified on an appropriate column (*e.g.*, a presto desalting column from Pierce). *See also*, Hawkin *et al.* (1992) *J. Mol. Biol.* 226, 889-896.

In one embodiment, poly-l-lysine is coupled to the protein (*e.g.*, where the protein is transferrin which binds the transferrin receptor), or is itself used as a targeting agent. Poly-l-lysine or poly-l-lysine-transferrin which has been linked to defective adenovirus mutants is delivered to cells with transfection efficiencies approaching 90% (Curiel *et al.* (1991) *Proc Natl Acad Sci USA* 88:8850-8854; Cotten *et al.* (1992) *Proc Natl Acad Sci USA* 89:6094-6098; Curiel *et al.* (1992) *Hum Gene Ther* 3:147-154; Wagner *et al.* (1992) *Proc Natl Acad Sci USA* 89:6099-6103; Michael *et al.* (1993) *J Biol Chem* 268:6866-6869; Curiel *et al.* (1992) *Am J Respir Cell Mol Biol* 6:247-252, and Harris *et al.* (1993) *Am J Respir Cell Mol Biol* 9:441-447). Adenovirus-poly-l-lysine-DNA conjugates bind the normal adenovirus receptor and are subsequently internalized by receptor-mediated endocytosis. Herpes viruses have similar properties. Transferrin-poly-l-lysine conjugates enter cells which comprise transferrin receptors. *See, e.g.*, Curiel (1991) *Proc. Natl. Acad Sci USA* 88: 8850-8854 and Wagner *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 89:6099-6013.

Expression Cassettes

The vectors of the invention are typically targeted against mammalian cells. However, because the vectors can be targeted against essentially any cell by the selection of the targeting agent, the choice of nucleic acids to place within any expression cassette encoded by the vector depends upon the intended target cell.

Examples of cells which can be transformed with the vectors of the invention include bacteria, yeast, plant, filamentous fungi, insect and vertabrate cells such as mammalian cells. It is expected that those of skill in the art are knowledgeable in the numerous expression systems available for cloning and expression of nucleic acids. Sambrook, Ausbel, and Berger provide a guide to expression cassettes. This ability to transduce cells makes the vectors of the invention generally useful as recombinant vectors for transducing cells with nucleic acids. One of skill will recognize the general need for vectors as tools for recombinant procedures.

Single-Chain Antibody Targeting

Cloned single chain antibody molecules can be generated by PCR from the variable region of known antibodies where a hybridoma is available, using standard techniques. This technology is adapted to target a viral vector against essentially any protein of interest. For example, fusions between a viral surface protein, such as a retroviral glycoprotein, an adenoviral coat protein or the AAV cap protein, and a single chain antibody are provided. A fusion protein strategy is favorable since it simplifies large-scale vector production once a vector design is established.

As an exemplar strategy, developed single-chain variable region antibodies (ScAb) against the CD34 marker on stem cells were developed. FACS binding experiments were performed showing that the ScAbs specifically bind to the CD34 marker. In addition, ScAbs were fused to the N-terminus of AAV Vp2 and used to generate AAV particles in packaging experiments. Furthermore, these particles had elevated levels of transduction on cell lines expressing CD34 (KG-1) compared to control vector. FACS binding experiments were performed to demonstrate that the ScAb-vector was able to specifically bind to the CD34 marker. Anti-sera against the ScAb are used to directly detect the presence of the ScAb on intact particles.

In one embodiment, the construct described above was made using a wild type (WT) rAAV in the construction of vector comprising the ScAb, so that the resulting targetable vector was dual-tropic as it was able to bind to both the AAV receptor and to the CD34 marker. A more preferred targetable vector is defective in normal AAV binding to avoid significant dilution by such a dual-tropic vector upon injection into a human. For example, the ScAB is cloned into the cap protein of an

AAV binding mutant such as one exemplified herein, which has at least a 100-fold decrease in binding affinity compared to the WT.

In one embodiment, a nucleic acid encoding the ScAb was fused to the Vp2 region of AAV. *See*, the Examples, *supra*. Other constructs include the ScAb nucleic acid fused to the N-terminus of Vp1 or Vp3, C-terminal addition of ScAb to various cap proteins, and adding ScAb at the site of the mutation. Vectors which contain all of the capsid elements in cis are desirable, although vectors systems delivering the ScAb-Vp2 in trans are also provided.

In ScAb fusions, a hinge region (derived from a native immunoglobulin) is preferably provided at the site of attachment of the ScAb. This hinge region increases the flexibility of fusion proteins comprising single chain antibodies and facilitates maintenance of native conformations for the domains of the fusion protein. All constructs are optionally evaluated both for their ability to express the ScAb-capsid fusion protein, generate vector particles, and for the presence of the ScAb domain on the surface of the vector particle. Recombinant vector constructs are assessed for their ability to bind to specific target cells and for transducing activity.

One new target cell line provided herein is a Cos cell line expressing CD34. It was found that the common $CD34^+$ cell line, KG-1, appeared to have a defect in its ability to be transduced by AAV relative to non-target cells such as HeLa cells. Cos cells are readily transducible by AAV and exhibit low native cross reactivity to CD34 antibodies.

Library Technology

One design consideration for making targetable vectors is determining the optimal placement of the targeting ligand within a surface protein on a vector of the invention. The size of a targeting ligand such as an ScAb is also relevant as the size of the targeting ligand has an impact on the overall conformation of vector proteins which are required for correct vector assembly.

Although the receptor for AAV has recently been identified, there is no information available concerning its normal cellular function, interaction with infectious virions, and mechanism of virus internalization. In spite of these unknowns, as indicated *supra*, regions on AAV cap proteins that influence AAV receptor binding are identified herein. Cap fusion proteins which allow targeting of AAV to CD34 cell surface proteins with subsequent transduction of specific target

cells are provided. However, a strategy for optimizing these vectors is also needed, and provided herein.

A strategy which allows for unbiased construction of vectors capable of targeting and efficiently transducing any cell type of interest is provided herein. Both library technology and *in vitro* evolution of candidate vectors are utilized so that vectors are randomly selected for that have optimal transduction capacity with respect to both receptor binding and gene delivery. This system is specifically designed within the constraints of limited available information regarding vector structure and internalization processes. Furthermore, this system is amenable to virtually any vector and any target cell, even if a candidate receptor for that cell has not been identified.

The library strategy has two basic components: 1) identification of small targeting peptides by phage display libraries, and 2) randomized insertion of those identified peptides in vector surface proteins followed by amplification and evolution viable vector particles. For simplicity, the following discussion relates to this strategy for developing AAV vectors; however, the strategy is applied to essentially any vector, including retroviral vectors, adenoviral vectors, parvoviral vectors and the like.

In one embodiment, the targeting strategy described herein provides for the isolation of a viral vector having a targeting ligand which binds to a cell receptor such as CD34 or CD4. Typically, the vector is made by isolating a bacteriophage particle which specifically binds to a cell comprising a selected receptor such as CD34 or CD4 from a random phage display library, subcloning a subsequence derived from the identified bacteriophage particle corresponding to the targeting ligand into a vector nucleic acid, and expressing the vector nucleic acid in a cell, thereby making a targeting protein comprising the targeting ligand. The targeting ligand is packaged into the vector, *e.g.*, in a packaging cell which comprises additional vector components. The additional components are optionally supplied in *trans*, *i.e.*, by *trans*-complementation as described, *supra*. Optionally, one or more additional viral component is encoded in the vector nucleic acid, in addition to the targeting ligand. In general, the targeting ligand is cloned into a viral surface protein, such as an AAV or Ad capsid protein, or a retroviral envelope protein, or the like, as described herein.

Ligand Identification and Development

As previously stated, a vector targeting system contains a cell surface target-binding component (ligand) which can be associated with the vector surface protein (*e.g.*, AAV cap protein) and which redirects the vector to a new cell surface target (receptor) of choice. Ideally, the ligand is inserted into the capsid in the form of a fusion protein; however, some currently available candidate ligands for desired receptors are large in size (*e.g.*, ScAb) and thus may not be optimal due to the influence of the ligand on the overall structure of the virion capsid assembly. Consequently, for generation of fusion proteins, it is advantageous to use small peptides (~20 amino acids) so that incorporation into the capsid protein results in minimal changes to the overall conformation of the virion.

Phage display libraries (available, *e.g.*, from Affymax in Santa Clara, CA), are used for selection of ligands with unique and high binding affinities for specific cell types. These libraries are used both for the selection of ligands capable of binding to specific receptors of choice (*e.g.*, CD34, CD4, *etc.*) and ligands capable of binding to specific cell types of interest (*e.g.*, hematopoietic stem cells, T-lymphocytes, *etc.*) where a target receptor has not been identified. The later situation occurs, for example, where true progenitor cells may be identified as CD34+ CD38- as opposed to CD34+ CD38+, with the only known difference in the receptor profile of these cells being the lack of the CD38 marker in the target true progenitor cell, making targeting to these cell more difficult using previous techniques.

Phage display libraries containing randomized peptides (*e.g.*, 20 mers, or similar sized peptides) are selected against a target cell type, such as primary $CD4^+$ T-cells, $CD34^+$ stem cells or $CD34^+$ $CD38^-$ stem cells. Briefly, the cell type used for selection is incubated with phage library at a concentration of 1e5 cells/3e9 phage particles in 2 ml of PBS/Ca^{++}/ BSA (standard incubation buffer). at 37°C.' After washing, the cells are lysed and the liberated phage particles are amplified in bacteria under standard conditions. The above process is repeated about 3 times, followed by sequencing of the selected clones (*See* Figs. 1 and 2).

As an empirical matter, a single peptide species is typically isolated by this technique which is specific for the target cell in question, rather than broadly reactive for many cell types. Presumably this is because the starting libraries are

substantially under-representative of all possible combinations for randomized 20-mers, thus many species are not contained within any given library. In addition, factors which influence high affinity binding and selection for a given peptide are apparently cell type specific. This may be due to receptor abundance or other factors which influence the overall conformation of surface proteins of a given cell type. For example, there are subtle differences in the same protein on different cell types which can be rendered targetable by the phage system. Alternatively, the phage system produces peptides which recognize combinations of surface proteins that have unique quaternerary structure on a given cell type.

Once a peptide is identified it is tested for specific binding by adding a fixed concentration of peptide-phage species in the presence of increasing concentration of purified homologous free-peptide. The cell associated phage is then recovered and titered by standard assays and the resulting affinity value is calculated by Scatchard analysis. Similar experiments are performed on typically at least 5 different unrelated cell types to demonstrate the specificity of the isolated peptide. Most undesirable targets are essentially ubiquitous (*e.g.*, the insulin receptor) and examination of 5 unrelated cell types should permit elimination of peptides which bind to these common receptors.

In the case of peptides against CD34+ CD38- cell types, the library is selected against $CD34^+$ $CD38^-$ cells at low stringency, and the resulting bound material is selected against $CD34^+CD38^+$ cells at high stringency. The eluate from the latter pass (unbound material) is substantially free of phage which bind to $CD34^+$ $CD38^+$ cells and can thus be selected against $CD34^+$ $CD38^-$ cells at high stringency.

Peptides which are specific for the desired cell type, relative to the unrelated cell types and which have K_d values greater than or equal to 1e-6 M, are mutagenized in a new library with homology to the isolated peptide sequence(s). This new library is subjected to an analysis identical to the above with the goal of isolating related peptides with K_d values of greater than or equal to 1e-9 M. The advantages of this procedure is that binding peptides are made against cells without concern or knowledge of the receptor profile of the given cells. One disadvantage is that even though the peptides are screened for lack of binding against, *e.g.*, five other cell types, it is not certain what the specificity of this peptide is, since its binding target is unknown and there is usually no foundation in the literature to help establish the tissue distribution of the unidentified receptor. Some of these concerns

are minimized by testing in animals (*e.g.*, SCID mice) and empirically determining the tissue distribution of an injected vector comprising the targeting peptide. In addition, if a particular peptide is identified which has an especially low K_d (*i.e.*, 1e-11 M) it is desirable to perform cross-linking experiments to identify the binding protein in parallel, or prior, to initiating animal studies.

In the alternative strategy, peptides contained in phage display libraries are will be selected against purified binding proteins of interest (*i.e.*, a CD34 protein). For example, methods of screening peptides bound to a solid matrix is known. The peptide phage that are isolated by this analysis are then be screened against cells of interest that contain the protein in question (*i.e.*, CD34 expressing cell lines). This pre-selection step with a purified protein limits the library to the binding protein of interest, and the subsequent selection steps on intact cells isolates phage which bind to regions of the protein that are exposed on the cell surface. Similar to above, the isolated peptide are mutagenized with the goal of obtaining a peptide with a Kd of at least 1e-9 M.

In another variation, cell lines which express the protein of interest (*i.e.*, CD4, CD34) may be used for subtractive purification of binding phage, provided that an identical parent cell is available which does not express the given protein. Such cell lines are prepared by transfection or by infection with common expression vectors such as vaccinia. The phage are bound to the null cell line to remove phage that bind to proteins common to the two cell lines, and then bound and eluted from the target cell line. All peptides obtained by this and the above procedures, and with the appropriate K_d, are used to construct targetable vector libraries as outlined below.

Identifying ligand(s) bound to cell surface CD4 molecules is one of the important tasks in developing a targetable vector that can specifically deliver therapeutic genes into human CD4+ T-lymphocytes. Phage display technology was used as described to explore human CD4 binding peptides from a random peptide library as candidate binding ligands to human CD4. The following are examples of CD4 binding peptides that were identified: GAVQPRGATSKLYLLRMTDK; MGEKLHRVHIRTNTPSVYSR; LEPRVAQRGQMVKFTYMRLP; and HAWWKPWGWSIEALAPTAGP.

Using phage binding assay, we have shown that these peptides specifically bind to the purified soluble CD4 molecules and CD4+ cells, suggesting a specific binding to cell surface CD4 molecules. We have also demonstrated that the phages carrying these peptides can be internalized into CD4+ T-cells upon binding, which makes these peptides, or their modified versions, ideal candidate ligands for targeting human CD4 T-cells in a targetable vector setting. Thus, these peptides are useful as epitopes expressed on the surface of a vector to permit the vector to transduce a CD4 cell, *in vitro*, *ex vivo* or *in vivo*. This has general utility in recombinant techniques for transduction of CD4 cells, and in therapeutic techniques where the transduction of CD4 cells by a therapeutic vector is desired, such as in the case of HIV infection.

After identification of candidate binding ligand with high affinity, it is not known *a priori* if that particular ligand, in the context of a vector surface fusion protein, will allow for appropriate vector entry and uncoating. For example, pathways of ligand internalization may be non-existent or may be by a mechanism substantially different from normal virus receptor-mediated internalization. Conversely, the mechanism of normal viral internalization may be by capsid components which are completely receptor/ binding ligand independent. This latter point is supported by examples of some viral systems (*e.g.*, adenovirus and HIV), where independent accessory proteins mediate viral entry (*See also*, Fig. 3) To a certain extent, these issues are determined empirically by the procedures described in the following sections. However, it is desirable, albeit not necessary, to have a rapid screen to eliminate ligands which do not mediate appropriate vector internalization. A description of such a procedure is described in the section referred to as "Modification of Pre-Formed Vector Particles, below".

Targetable Vector Libraries

Targetable vector libraries are constructed in AAV vector binding mutants as identified *supra*. The goal is to generate a vector library with a ligand of interest inserted at random locations within the cap coding sequence. This library is amplified in host cells so that rare vectors, which have the optimal insertion point in the cap coding region for the ligand and forms a functional vector particle, will be enriched.

Construction of these libraries is performed, *e.g.*, in the following 7 phases:

Phase 1: Subcloning of binding mutant cap region into a small plasmid backbone; Phase 2: Random linker insertion into cap region to construct a cap linker library; Phase 3: Subcloning of the binding ligand coding sequence into a linker library to construct a cap ligand library; Phase 4: Subcloning the cap ligand library into a WT AAV background to construct the targetable vector library; Phase 5: Packaging of the targetable vector library; Phase 6: Infection of target cells with targetable vector library followed by amplification, enrichment, and *in vitro* evolution of targetable vector; and, Phase 7: sequencing of the enriched vector population. These phases are described graphically in Fig. 4.

Phase 1: Subcloning of binding mutant cap region into small DNA plasmid

This phase isolates the cap coding sequence within a relatively small DNA plasmid so that subsequent random linker insertion steps, described below, are concentrated on the cap sequence, rather than on irrelevant flanking sequences. In this phase, a cap sequence which is binding deficient as described, *supra*, is cloned into a plasmid using standard techniques. *See*, Sambrook, Ausubel, and Berger *supra*. Similarly, this step is performed on surface proteins from other viruses, such as a retroviral *env* protein, or an adenoviral capsid protein.

Phase 2: Random linker insertion into cap region to construct cap linker library

The purpose of this phase is the insertion of a linker sequence into the cap portion of the cap gene (or other surface protein) in the plasmid constructed in phase 1, to provide unique restriction sites for cloning of the desired ligand. The linker will ultimately be less than 20-30 nucleotides; however, initially a removable selectable marker is included in the linker so that recombinants containing linker sequences can be easily selected for, thereby increasing the frequency of linker containing molecules in the library. In parallel procedures, a hinge region of approximately 30 nucleotides is optionally included in the final linker. This hinge region increases the flexibility at the site of ligand addition and reduces the impact of the ligand on the final conformation of the cap (or other surface) protein, thereby facilitating vector packaging functions of the protein. A parameter in the construction of the library is the degree of randomness that is achieved, with the ideal situation being a library that contains a linker insertion at every base pair

within the nucleic acid encoding the cap (or other surface) protein. Three strategies are optionally analyzed for their ability to produce randomized linker libraries within the cap sequence: 1) partial restriction digest 2) chemical mutagenesis and 3) transposon-based mutagenesis.

In the first strategy, a partial restriction digestion will be performed on the cap sequence using a restriction enzyme, or multiple enzymes that recognize(s) a four base pair sequence (*i.e.*, a frequent cutter). The conditions are optimized such that digestion results in individual full-length molecules cut at a single site. The linker is then added by cohesive end ligation using sites at the end of the linker that are compatible with the restriction enzyme used in the partial digestion. An advantage of this procedure is its simplicity. A disadvantage is that it an optimal degree of randomness may not be achieved, as it will be difficult to find restriction enzymes which cut frequently enough.

In an alternative strategy, the DNA encoding the cap (or other surface) protein is treated with formic acid, which results in de-purination of the DNA to create a single stranded nick at random locations. The DNA is then treated with exonuclease III to cut both strands of the molecule at the site of the nick. In the process of digesting the nick, exonuclease III also cuts back from 0-30 nucleotides so that complete randomness with respect to the site of the cut is theoretically possible. Blunt-end ligation of the linker is used to produce closed circles. The advantage of this procedure are that it is highly random. A disadvantage is that the chemical treatment and conditions for exonuclease III digestion may need extensive optimization. This procedure and the partial digestion procedure are outlined in more detail in Sambrook, *supra*.

A final strategy is to use transposon-based mutagenesis to directly insert the linker into random locations within a DNA encoding the cap or other surface protein. In this procedure, the cap plasmid is exposed to a transposon such as At-2 which contains the dHFR gene (*see*, Fig. 5). This reaction is performed entirely *in vitro* using Ty1 integrase provided from Ty1 virus-like particles. Conditions are optimized to maximize the frequency of insertion, which can achieve the theoretical maximum of one insertion per base pair. *See*, Devine and Boeke (1994) *Nucleic Acids Research* 22(18):3765-72. The mutagenized DNA is transformed into *e. coli* and selected first for ampicillin resistance and subsequently

selected for resistance to methopterin. The frequency of recombinants is typically around 1 amp^r Tmp^r recombinants/10e4 amp^r transformants; thus, this selection step dramatically increases the representation of recombinants in the final library. Unique restriction sites are available for removing the dHFR gene so that approximately 30 nucleotides are left behind as a linker. This procedure is particularly advantageous because the insertional mutagenesis reaction is a standard reaction which is as easy to perform as a routine restriction digest (a beta-test version of a kit from Perkin-Elmer can be obtained to facilitate the reaction). A disadvantage is that some effort must be made to ensure a maximal level of random insertion frequency.

Each of the above procedures can be analyzed for the degree of randomness in the insertion of the linker by digesting the mutagenized DNA population with a unique restriction enzyme that lies outside of the cap gene (*i.e.*, in the vector plasmid) followed by end-labeling of the DNA with ^{32}P. The DNA is digested with a restriction enzyme that cuts within the linker sequences. Subsequently, the end-labeled DNA is run on a sequencing gel, and under optimal conditions and a "ladder" pattern indicates the degree of randomness. Optimally, the ladder should have a one base pair separation. In addition, the intensity of each band will vary according to its relative abundance, such that under optimal conditions they will be of equal intensity. Different restriction enzymes can be chosen to generate the site of end-labeling so that the insertion points can be analyzed by "walking" down the entire gene (this step is necessary where the resolution capacity of the sequencing gel prohibits analysis of the entire length of the plasmid in a single run). Furthermore, theoretical maximum sizes can be calculated so that only insertions within the cap coding sequence (or other surface protein coding sequence) is assessed. The degree of randomness from each analysis is calculated by determining the mean intensity of the bands and counting the number of bands that fall within 20% of this intensity. The sum of this value is equal to the degree of randomness (dR). Each run on the sequencing gel would be expected to generate a theoretical maximum dR value of 300-400 as this is the typical resolution capacity of a standard sequencing gel. The total theoretical maximum dR value for cap should be approximately 2500 as this is the length of the cap coding region. The library which is generated by the above procedures which contains the highest degree of randomness is used in Phase 3.

Phase 3: Subcloning of binding ligand coding sequence into linker library to construct cap ligand library

The primary consideration for this portion of the project is the choice of ligand. Several procedures are described *supra* for ligand selection. Three example ligands include 1) anti-CD34 ScAb used in the examples, *supra*; 2) ligands generated from phage display libraries, and 3) the HIV C4 region of the HIV envelope protein.

1) Although success has been achieved by using the anti-CD34 ScAb in the AAV vectors described *supra*, it is possible to optimize any recombinant AAV (rAAV) vector for replication competence and target cell transducing ability. Improvements to the current targetable vectors with this new targeting strategy are contemplated.

Cloning of this ligand is performed using a PCR strategy in which the appropriate restriction sites are placed on the ends to allow for insertion into the linker library.

2) The criteria for selection of ligands from the phage display libraries have been outlined above. The ligand that is available with the optimal K_d value is selected for subcloning into the linker library. Once this sequence has been identified, an oligo corresponding to this sequence is synthesized which contains the appropriate restriction sites for cloning.

3) Robey *et. al.* (1995) *Journal of Biological Chemistry* 270(41):23918-21 have identified a 17 amino acid region on HIV Gp120 that is capable of specific binding to CD4 with a K_d value of 8.5e-9. This peptide (termed the C4 peptide) is suitable for targeting $CD4^+$ cells and is optimized using the strategy outlined herein. In addition, the peptide is used as a starting material for phage display libraries with the intention of improving its K_d value for CD4, as described, *supra*. Cloning of this molecule or modified forms is as described for the random phage display library ligand. Because it is readily available, the C4 peptide is also tested on the N-terminus of Vp1 and Vp2 in WT AAV, similar to the tests of ScAb described, *e.g.*, in the examples. This allows for a quick screen of C4 utility in AAV vector targeting.

Phase 4: Subcloning cap ligand library into WT AAV background to construct targetable vector library

Sub-cloning of the cap ligand library of interest is performed into a WT AAV background. Briefly, the ligand library is digested with appropriate restriction enzymes to release the modified cap (or other surface protein) coding DNAs which are then "shot-gun" cloned into an otherwise WT AAV background. Since the plasmid cap library contains one of the previously identified cap binding mutations which replaces the wild-type AAV cap protein, the intention is to restore binding and replication competence by addition of a foreign ligand to the resulting AAV vector. Using a replication competent vector allows for amplification of rare recombinants that have the appropriate cap conformation. In addition, chemical mutagenesis or irradiation of target cells can be included during the amplification phase so that random mutations can be introduced to optimize and improve the infectivity of virions containing viable insertions.

Phase 5: Packaging of targetable vector library

Packaging of the library is by appropriate standard vector packaging methods, depending on the particular vector employed.

Phase 6: Infection, amplification, and evolution of targetable vector library

For AAV, target cells of interest are infected with the vector library in the presence of a helper virus such as adenovirus under standard conditions. After maximum cytopathic effect, the cells are harvested and the resulting lysate is used in a subsequent round of infection. This procedure is repeated 3-6 times. In parallel, a similar experiment is performed, with the exception that during the replication phase of the virus, the cells are mutagenized by irradiation under standard conditions or by treatment with chemical mutagens. The purpose of this mutagenesis phase is to increase the rate of evolution of the virus and subsequent enrichment of forms optimized for infectivity and replication via the target receptor. Vectors which initially have sub-optimal cap conformations gradually acquire random mutations which confer a selective advantage for growth in a given target cell.

In the case of a targeted protein such as a cellular receptor, the chosen cell type for amplification can be any cell or cell line which expresses that protein. If a suitable cell which stably expresses the protein cannot easily be made then a vaccinia virus vector is used both for target protein expression and helper function for AAV. In the case of a targeted cell, the cell that was used in the phage display library selection are used in this experiment as well. Target cells are

typically permissive for replication of AAV helper virus (*i.e.*, adeno, herpes, vaccinia), or recombinantly express the necessary AAV helper components .

Phase 7: Sequencing of selected vector

Following Phase 6, the material from the final lysate is PCR amplified using primers for the distal 5' and 3' ends of cap. The amplified material is sub-cloned into a standard TA cloning vector and, following transformation, approximately 50-100 colonies are selected for subsequent analysis. Initially, plasmid DNA is prepared and digested to verify that the ligand has been retained within the cap coding sequence (preliminary information can be obtained at this point as to the degree of heterogeneity of the processed library). All positive clones are sequenced using a primer specific within the linker sequence with the goal of identifying flanking sequences which specify the insertion point of the ligand. New primers are selected for any sequencing reactions that do not work, as it is assumed that significant mutation has taken place at the ligand locus, thereby preventing binding of the primer. Using the sequence data, groupings containing 100% similarity are made based on the number of unique sequences. These groupings are only an estimate of similarity/homology since other important mutations outside the region sequenced can be introduced during the amplification phase. This entire process is improved by incorporation of high-throughput sequencing technology.

One clone within each group is packaged and tested for replication competence on the target cell line of interest, *e.g.*, by slot blot analysis for replicating genomic DNA. The most replication competent clone is sequenced in its entirety and used to construct an AAV targetable vector packaging plasmid (or other vector, as appropriate) and subsequently used to produce recombinant targetable AAV (rtAAV). In addition, if sequencing of the ITRs show differences relative to the WT, these new ITRs are tested in the genomic portion of the vector. Once final vector candidates are identified and used to produce targetable AAV vectors, transduction analysis is performed both on cells which contain the targeting receptor of choice and on cells which contain only the WT AAV receptor. Mixing experiments are performed to verify that the vector is capable of selectively transducing a small population of target cells in the presence of a high background of non-target cells.

Binding mutations

A number of cap mutants are shown *supra*. Binding mutations that have been identified to form particles and disrupt binding as measured by the b-gal binding assay are summarized in Fig. 6.

All of the mutations in Fig. 6 represent point mutations or small deletions. This was done intentionally to minimize the potential for pleiotropic effects. However, maximal mutations are also desirable as they potentially allow for larger ligands to be inserted, if the overall size of the given cap (or other surface) protein is limiting. The deletions are optionally maximized and the effect of increasing the length of the mutation determined on the phenotype of the vector as measured by previously established assays. Once these additional mutants are identified they are used as a source for DNA encoding the cap (or other surface protein) into which a ligand is cloned.

New mutants are generated by random linker insertion into a DNA encoding a WT cap (or other WT surface protein) similar to strategies outlined for the targetable AAV vector. If the C4 peptide is verified to allow for targeting in the WT AAV vector (*see*, Targetable Vector Library, Phase 3), then this could be used as a "test" ligand for generating new binding mutants. Hence, the C4 peptide would be cloned into the N-terminus of Vp1 and Vp2 prior to linker insertion mutagenesis. Following mutagenesis the resulting binding mutant library is packaged and bound to HeLa cells in the absence of adenovirus to remove any WT receptor binding AAV. Unbound material is then used to infect HeLa CD4 cells in the presence of adenovirus, and amplified 3-6 times. After each round of amplification, the lysate is passed over normal HeLa cells to remove any WT binding AAV that may have developed. The final lysate is cloned and sequenced by PCR to determine the site of the binding mutations and presence of the C4 ligand. The appropriate clone is then tested for targeting and, subsequently, binding in the absence of C4 peptide. This binding mutant is used to construct future targetable vectors with ligands other than C4 and at more optimal ligand insertion points.

AAV serotype 4 was recently isolated at the NIH. Unlike AAV-2, which binds to a receptor that is essentially ubiquitous on animal cells, AAV-4 is very restricted in terms of its tropism. The only cells which have been identified as positive for AAV-4 infection are Cos cells and glial cells. Other standard cell lines for AAV-2 (*i.e.*, HeLa) are not infectable by AAV-4. Furthermore, because AAV-4 was originally obtained in nature from adenovirus infected monkeys, the

human population is seronegative for this virus. A recombinant vector system has been constructed from AAV-4 and is essentially identical to the AAV-2 system. In addition, titers from rAAV-4 are approximately 10-fold higher than AAV-2 in side-by-side comparisons. If AAV-4 can transduce either $CD4^+$ T-lymphocytes or $CD34^+$ stem cells, it may be used as a vector to transduce these important cell types. If AAV-4 is negative for transduction of these cell types, it is optionally used as an alternative to the AAV-2 binding mutants described *supra*. The advantages of using this vector are essentially three-fold: 1) because the WT AAV-4 vector can be used as a "natural binding mutant" concerns about the impact of mutations on the overall conformation of the particle and changes in adaptability for ligand insertion are eliminated; 2) any observable defect will solely be due to ligand insertion; and 3) the seronegative status of the human population for AAV-4 simplifies *in vivo* injection of the final vector.

As another alternative, the expression of the AAV-2 receptor is knocked-out by expressing a ribozyme in a cell which cleaves the mRNA for the receptor. Essentially any cell can have the AAV-2 receptor abrogated by transducing the cell with a vector encoding an appropriate ribozyme. Alternatively, AAV receptor null cells can be identified by FACS or other cell sorting techniques which measure the expression of the receptor (ELISA, western blotting, *etc.*) on the surface of the cell.

AAV-2 receptor null cells are used in a vector library systems as follows:

a) In the initial library strategy a potential concern with using binding mutants is that there is a chance that WT recombinants may form, or compensatory mutations may be introduced which restore WT binding. Because of the nature of the overall strategy, these recombinants or second-site mutants would be enriched and may mask enrichment of desired recombinants. Thus, cells which lack WT AAV binding capability would eliminate this concern.

b) In a similar strategy cells which do not bind WT AAV can be used to enrich a targetable vector within a library which is randomized both respect to the insertion point of the ligand and binding mutations. Hence, beginning with WT AAV cap, a cap vector will be constructed as described for the previous library. Linker insertion mutagenesis will then be performed on this vector. The idea of this particular insertion step is to create a random set of AAV binding knock-out

mutants. Following linker insertion, a targetable ligand is either added to the knock out linker, or a second round of linker insertion mutagenesis is performed followed by ligand addition. This new library is used to infect the AAV-2 receptor null cells which express the targeting protein of interest (*i.e.*, CD34). Similar to above, amplification and evolution is performed. Finally, the resulting vectors are screened for tropism, as some of the vectors that are identified by this procedure can be dual-tropic (*i.e.*, AAV receptor and CD34 binding). Dual tropic vectors are eliminated by screening on AAV-2 receptor positive cells.

This approach has the apparent limitation that only cells which lack the AAV receptor can be utilized in the amplification and evolution phase. This is not true, however: 1) Ribozyme vectors are constructed to knock out the AAV-2 receptor in any cell type and 2) if the initial amplification/evolution phase is performed in a standard cell line lacking the AAV-2 receptor, the resulting vector can be used as a true functional binding mutant for construction of subsequent libraries with other ligands of interest. Thus, the library technology based on an AAV-2 receptor null cell is adaptable and useful for generating additional AAV binding mutants.

Modification of Pre-Formed Vector Particles

It is desirable to create vectors which incorporate targeting ligands into their capsid protein as a part of the normal assembly process, rather than adding these components later as a modification to pre-formed particles. Vectors containing targetable capsid fusion proteins are more amenable to a reproducible and efficient production process. This approach may be helpful where the strategies outlined above are not optimal. Where generating targetable vectors via post-assembly modification is straightforward, this approach offers a method for rapidly testing ligand/receptor interactions in the context of internalizing a vector and delivering the trans-gene. This approach would thus be useful for analyzing candidate ligands for the fusion protein strategy discussed above, where concern exists that a particular ligand/receptor interaction may not allow for effective internalization of the vector. Several approaches for modifying pre-formed vector particles for purposes of targeting are outlined below.

The peptide display libraries that were previously described are used to generate bivalent peptides where one portion binds to AAV and the second portion binds to the target protein of interest. To accomplish this, two sets of

library panning experiments are performed, where the first experiment is to select peptides which bind to purified AAV particles and the second experiment, in parallel, selects binding peptides for the cell surface marker of interest. The sequencing information from these two experiments is used to generate a single linear peptide which has binding affinity for both AAV and the cell surface marker of interest. If the binding affinity for the AAV binding peptide is not sufficiently high to allow for reliable use of the complex as a targetable vector, then a cross-linking reaction can be performed to covalently attach these ligands to the vector.

Once the AAV binding peptide is identified, this method allows for rapid pre-screening of candidate ligands as vector targeting molecules prior to incorporating them into the various vector libraries described, *supra*. In addition, a bivalent peptide that is successfully used in this procedure can also be used to quickly test a panel of binding mutants for transducing function.

A similar method for modifying pre-formed vector particles is to utilize bivalent antibodies that have affinities for both AAV and the target receptor of choice. These antibodies are generated by chemically cross-linking monoclonal antibodies. Alternatively, streptavidin could be incorporated into the AAV particle either as a fusion protein or by cross-linkage directly to the AAV particle. Following this modification, biotinylated targeting ligands are added by direct non-covalent binding to streptavidin. In a variation to this technique, the AAV binding peptide technique that is described above may be modified so that the targeting portion of the peptide is not selected to bind to a specific receptor but rather will bind to biotin.

Cells Transformed by The Vectors of the Invention

The culture of cells used in conjunction with the present invention, including cell lines and cultured cells from tissue or blood samples, including stem cells is well known in the art. Freshney (*Culture of Animal Cells, a Manual of Basic Technique, third edition* Wiley-Liss, New York (1994)) and the references cited therein provides a general guide to the culture of cells. *See also*, Kuchler *et al.* (1977) *Biochemical Methods in Cell Culture and Virology*, Kuchler, R.J., Dowden, Hutchinson and Ross, Inc, and Inaba *et al.* (1992) *J. Exp. Med.* 176, 1693-1702.

Isolating Stem Cells

Hematopoietic stem cells are the primary targets for many forms of gene therapy, particularly gene therapy for HIV infection. Accordingly, stem cells are a preferred target for the vectors of the invention, particularly when the vectors encode anti-HIV agents. Many ways of isolating stem cells are known. In mice, bone marrow cells are isolated by sacrificing the mouse and cutting the leg bones with a pair of scissors. Stem cells are isolated from bone marrow cells by panning the bone marrow cells with antibodies which bind unwanted cells, such as $CD4^+$and $CD8^+$ (T cells), $CD45^+$ (panB cells), GR-1 (granulocytes), and Ia^d (differentiated antigen presenting cells). *For an example of this protocol see*, Inaba *et al.* (1992) *J. Exp. Med.* 176, 1693-1702.

In humans, bone marrow aspirations from iliac crests are optionally performed *e.g.*, under general anesthesia in the operating room. The bone marrow aspirations is approximately 1,000 ml in quantity and is collected from the posterior iliac bones and crests. If the total number of cells collected is $< 2 \times 10^8$/kg, a second aspiration using the sternum and anterior iliac crests in addition to posterior crests is performed. During the operation, two units of irradiated packed red cells are administered to replace the volume of marrow taken by the aspiration. Human hematopoietic progenitor and stem cells are characterized by the presence of a CD34 surface membrane antigen. This antigen is used for purification. After the bone marrow is harvested, the mononuclear cells are separated from the other components by means of ficol gradient centrifugation. This is performed by a semi-automated method using a cell separator (*e.g.*, a Baxter Fenwal CS3000+ or Terumo machine). The light density cells, composed mostly of mononuclear cells are collected and the cells are incubated in plastic flasks at 37°C for 1.5 hours. The adherent cells (monocytes, macrophages and B-Cells) are discarded. The non-adherent cells are then collected and incubated with a monoclonal anti-CD34 antibody (*e.g.*, the murine antibody 9C5) at 4°C for 30 minutes with gentle rotation. The final concentration for the anti-CD34 antibody is 10 μg/ml. After two washes, paramagnetic microspheres (Dyna Beads, supplied by Baxter Immunotherapy Group, Santa Ana, California) coated with sheep antimouse IgG (Fc) antibody are added to the cell suspension at a ratio of 2 cells/bead. After a further incubation period of 30 minutes at 4°C, the rosetted cells with magnetic beads are

collected with a magnet. Chymopapain (supplied by Baxter Immunotherapy Group, Santa Ana, California) at a final concentration of 200 U/ml is added to release the beads from the $CD34^+$ cells. Alternatively, and preferably, an affinity column isolation procedure can be used which binds to CD34, or to antibodies bound to CD34.

In another preferred embodiment, $CD34^+$ cells are isolated by peripheral blood leukapheresis after G-CSF mobilization.

Therapeutic Agents

Therapeutic agents of the invention, which are typically expressed by the expression cassettes of the invention, take several forms. Typically, the agent is a nucleic acid which has direct therapeutic activity, such as a replacement enzyme, molecular decoy, anti-sense RNA or ribozyme, or indirect anti-viral activity, *i.e.*, where the inhibitor *encodes* a direct anti-viral activity such as a therapeutic protein or suicide protein.

A variety of enzyme deficiencies are known to cause disease. *See,* Berkow (ed.) *The Merck Manual of Diagnosis and Therapy,* Merck & Co., Rahway, NJ; and Thorn *et al. Harrison's Principles of Internal Medicine,* McGraw-Hill, NY. For instance, insulin misregulation/deficiency causes diabetes. The present invention provides a treatment for such diseases in which a patient's abnormal cell type (*e.g.*, insulin deficient) is transfected with a vector of the invention. The vector comprises a nucleic acid encoding the deficient enzyme under the control of an appropriate promoter. The vector is targeted to the appropriate cell type using a targeting agent of the invention. For instance, where the vector expresses a streptavidin peptide which is bound to an antibody against pancreatic cells, the vector can be used to transduce pancreatic cells with a vector encoding insulin. Similarly, the combinatorial screening methods described herein can be used to identify targeting ligands which specifically hybridize to pancreatic cells. These targeting ligands are optionally recombinantly fused to a protein located on the surface of the vector where they are recognized by pancreatic cells, causing the cells to take up the nucleic acid associated with the vector.

Inhibitors: antisense nucleic acids, ribozymes, decoy nucleic acids and trans-dominant proteins

In many embodiments, the therapeutic agent inhibits growth of a target cell (*i.e.*, when the agent is an anti-cancer compound), or of a pathogenic virus (*e.g.*, HIV). Inhibitors are known in the art. The literature describes such genes and their use. *See, for example,* Yu *et al.*, *Gene Therapy*, 1:13 (1994); Herskowitz, *Nature*, 329:212 (1987) and Baltimore, *Nature*, 335:395 (1988). Inhibitors which are optionally incorporated into the expression cassettes of the invention include anti-sense genes, suicide genes, ribozymes, decoy genes, and transdominant proteins.

A suicide gene produces a product which is cytotoxic. In the vectors of the present invention a suicide gene is operably linked to an inducible expression control sequences which is stimulated upon infection of a cell by a pathogen such as HIV. Alternatively, the gene is operably linked to a constitutive promoter where the vector is used to transduce a pathogenic cell (*i.e.*, a cancer cell). Examples of suicide genes include thymidine kinase and cytosine deaminase. *See*, Huber *et al.* European Patent Application 95100248.4 and Mullen *et al.* U.S. Pat. 5,358,866.

An antisense nucleic acid is a nucleic acid that, upon expression, hybridizes to a particular RNA molecule, to a transcriptional promoter or to the sense strand of a gene. By hybridizing, the antisense nucleic acid interferes with the transcription of a complementary nucleic acid, the translation of an mRNA, or the function of a catalytic RNA. Antisense molecules useful in this invention include those that hybridize to oncogenic and viral gene transcripts. Two example target sequences for antisense molecules are the first and second exons of the HIV genes *tat* and *rev*. Chatterjee and Wong, *supra*, and Marcus-Sekura (*Analytical Biochemistry* (1988) 172, 289-285) describe the use of anti-sense genes which block or modify gene expression.

A ribozyme is a catalytic RNA molecule that cleaves other RNA molecules having particular nucleic acid sequences. General methods for the construction of ribozymes, including hairpin ribozymes, hammerhead ribozymes, RNAse P ribozymes (*i.e.*, ribozymes derived from the naturally occurring RNAse P ribozyme from prokaryotes or eukaryotes) are known in the art. Castanotto *et al* (1994) *Advances in Pharmacology* 25: 289-317 provides and overview of ribozymes

in general, including group I ribozymes, hammerhead ribozymes, hairpin ribozymes, RNAse P, and axhead ribozymes. Ribozymes useful in this invention include those that cleave viral transcripts, particularly HIV gene transcripts. Ojwang *et al.*, *Proc. Nat'l. Acad. Sci., U.S.A.*, 89:10802-06 (1992); Wong-Staal *et al.* (PCT/US94/05700); Ojwang et al. (1993) *Proc Natl Acad Sci USA* 90:6340-6344; Yamada *et al.* (1994) *Human Gene Therapy 1:39-45; Leavitt et al.* (1995) *Proc Natl Acad Sci USA 92:699-703; Leavitt et al.* (1994) *Human Gene Therapy* 5:1151-1120; Yamada et al. (1994) *Virology* 205:121-126, and Dropulic *et al.* (1992) *Journal of Virology* 66(3):1432-1441 provide an examples of HIV-1 specific hairpin and hammerhead ribozymes.

Briefly, two types of ribozymes that are particularly useful in this invention include the hairpin ribozyme and the hammerhead ribozyme. The hammerhead ribozyme (*see*, Rossie *et al.* (1991) *Pharmac. Ther.* 50:245-254; Forster and Symons (1987) *Cell* 48:211-220; Haseloff and Gerlach (1988) *Nature* 328:596-600; Walbot and Bruening (1988) *Nature* 334:196; Haseloff and Gerlach (1988) *Nature* 334:585; and Dropulic *et al* and Castanotto *et al.*, and the references cited therein, *supra*) and the hairpin ribozyme (*see, e.g.*, Hampel *et al.* (1990) *Nucl. Acids Res.* 18:299-304; Hempel *et al.*, (1990) European Patent Publication No. 0 360 257; U.S. Patent No. 5,254,678, issued October 19, 1993; Wong-Staal *et al.*, PCT/US94/05700; Ojwang et al. (1993) *Proc Natl Acad Sci USA* 90:6340-6344; Yamada *et al.* (1994) *Human Gene Therapy 1:39-45; Leavitt et al.* (1995) *Proc Natl Acad Sci USA 92:699-703; Leavitt et al.* (1994) *Human Gene Therapy* 5:1151-1120; and Yamada *et al.* (1994) *Virology* 205:121-126) are catalytic molecules having antisense and endoribonucleotidase activity. Intracellular expression of hammerhead ribozymes and a hairpin ribozymes directed against HIV RNA has been shown to confer significant resistance to HIV infection.

The typical sequence requirement for cleavage by a hairpin ribozyme is an RNA sequence consisting of NNNG/CN*GUCNNNNNNNN (where N*G is the cleavage site, and where N is any of G, U, C, or A). The sequence requirement at the cleavage site for the hammerhead ribozyme is an RNA sequence consisting of NUX (where N is any of G, U, C, or A and X represents C, U or A). Accordingly, the same target within the hairpin leader sequence, GUC, is targetable by the hammerhead ribozyme. The additional nucleotides of the hammerhead

ribozyme or hairpin ribozyme which mediate sequence specificity, are determined by the common target flanking nucleotides and the hammerhead and hairpin consensus sequences.

Altman (1995) *Biotechnology* 13: 327-329 and the references therein describe the use of RNAse P as a therapeutic agent directed against flu virus. Similar therapeutic approaches can be used against other viruses as well.

In preferred embodiments, the inhibitors of the invention include anti-HIV ribozymes, such as hairpin ribozymes (*see*, Wong-Staal *et al.* WO 94/26877 and PCT/US94/05700 and the references therein; *see also*, Yu *et al.* (1993) *PNAS* 90: 6340-6344; and Yu *et al.* (1995) *Virology* 206: 381-386), hammerhead ribozymes (*see*, Dropulic *et al.* (1992) *Journal of Virology*, 66(3):1432-1441), and RNAse P (*see*, Castanotto *et al.* (1994) *Advances in Pharmacology* Academic Press 25: 289-317). These ribozymes are constructed to target a portion of the Rev-binding virus' genome or nucleic acid encoded by the genome. Preferred target sites in HIV-1 include the U5 region, and the polymerase gene.

A decoy nucleic acid is a nucleic acid having a sequence recognized by a regulatory nucleic acid binding protein (*i.e.*, a transcription factor, cell trafficking factor, *etc.*). Upon expression, the transcription factor binds to the decoy nucleic acid, rather than to its natural target in the genome. Useful decoy nucleic acid sequences include any sequence to which a viral transcription factor binds. For instance, the TAR sequence, to which the tat protein binds, and the HIV RRE sequence to which the rev proteins binds are suitable sequences to use as decoy nucleic acids.

A transdominant protein is a protein whose phenotype, when supplied by transcomplementation, will overcome the effect of the native form of the protein. For example, *tat* and *rev* can be mutated to retain the ability to bind to TAR and RRE, respectively, but to lack the proper regulatory function of those proteins. In particular, rev can be made transdominant by eliminating the leucine-rich domain close to the C terminus which is essential for proper normal regulation of transcription. Tat transdominant proteins can be generated by mutations in the RNA binding/nuclear localization domain of Tat.

Examples of antisense molecules, ribozymes and decoy nucleic acids and their use can be found in Weintraub, *Sci. Am.*, 262:40-46 (Jan. 1990);

Marcus-Sekura, *Anal. Biochem.*, 172:289-95 (1988); and Hasselhoff *et al.*, *Nature*, 334:585-591 (1988).

In Vitro Cell Transformation

The vectors of the invention are useful for *in vitro* cell transformation. The ability to transform cells is of general commercial importance in biological manufacturing, drug screening assays, cloning procedures and the like. For instance, the vectors of the invention optionally comprise a mucleic acid which encodes a commercially valuable protein such as insulin, TPA, erythropoietin, *etc.* The vector is used to transform a cell, which expresses the protein.

Ex Vivo Therapy

The vectors of the invention are useful as cloning vectors for gene transfer *in vitro*. In addition, the vectors are useful as gene therapy vectors in both *ex vivo* and *in vivo* procedures. *Ex vivo* methods of gene therapy involve transducing a target cell *ex vivo* with a vector of this invention, and introducing the cell into the organism. Target cells are selected based upon the range of the targeted vector. *See, e.g.*, Freshney *et al.*, *supra* and the references cited therein, and the discussion provided herein for a discussion of how to isolate and culture cells from patients. Alternatively, the cells can be those stored in a cell bank (*e.g.*, a blood bank).

Thus, for example, a patient infected with a virus such as HIV-1 can be treated for the infection by transducing a population of their cells with a vector of the invention and introducing the transduced cells back into the patient as described herein. Thus, the present invention provides a method of protecting cells from infection *in vitro*, *ex vivo* or *in vivo*.

In Vivo Therapy

Current protocols for clinical use of gene therapy vectors typically involve removal of the target tissue from the patient, transduction of the trans-gene, and re-infusion of the modified cells. In some isolated cases it has been suggested that the therapeutic vector could be directly delivered to the target tissue by surgical means, or by aerosol in the case of the lungs. Clearly, for sake of its simplicity, a non-surgical and broadly applicable method for direct delivery of the vector in vivo would be favored over ex vivo therapy. Moreover, maintaining the target cells in vivo throughout therapy prevents undesired alterations in their phenotype which may

occur during *ex vivo* approaches, and thereby enhance the reconstitution properties of the transduced cells. For example, in the case of HIV infection it would be advantageous to directly inject therapeutic vectors intravenously and obtain targeting of both CD4+ cells and stem progenitor cells. Thus, there is no need to place these cells in an artificial *ex vivo* environment which may significantly influence their ability to respond to various stimuli and inhibitors that are part of normal immune function and/or stem cell development. Furthermore, this dual approach to in vivo targeting for HIV infection would be ideal as it eliminates concerns about the origin of CD4+ cells in HIV+ adults (*i.e.*, from stem cells or by proliferation of existing CD4+ cells).

Vectors of the invention can be administered directly to the organism for transduction of cells *in vivo*. Administration of vectors comprising the therapeutic agents of the invention, and cells transduced with the gene therapy vectors is by any of the routes normally used for introducing a molecule into ultimate contact with blood or tissue cells.

The vectors or cells are administered in any suitable manner, preferably with pharmaceutically acceptable carriers. Suitable methods of administering such vectors or cells in the context of the present invention to a patient are available, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions of the present invention.

Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the vector dissolved in diluents, such as water, saline or PEG 400; (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as liquids, solids, granules or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, tragacanth, microcrystalline cellulose, acacia, gelatin, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, stearic

acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise the active ingredient in a flavor, usually sucrose and acacia or tragacanth, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the active ingredient, carriers known in the art.

The vectors, alone or in combination with other suitable components, can be made into aerosol formulations to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

Suitable formulations for rectal administration include, for example, suppositories, which consist of the vector with a suppository base. Suitable suppository bases include natural or synthetic triglycerides or paraffin hydrocarbons. In addition, it is also possible to use gelatin rectal capsules which consist of a combination of the vector with a base, including, for example, liquid triglyercides, polyethylene glycols, and paraffin hydrocarbons.

Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives.

Intravenous administration is the preferred method of administration for gene therapy vectors and transduced cells of the invention. The formulations of vector can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and in some embodiments, can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, for injections, immediately prior to use. For many vectors, this mode of administration will not be appropriate, because many particles are destroyed by lyophilization. Some vectors (*e.g.*, vectors utilizing an AAV capsid), however, tolerate lyophilization well.

Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. Cells transduced by the vector, *e.g.*, as described above in the context of *ex vivo* therapy, can also be administered parenterally as described above, except that lyophilization is not generally appropriate, since cells are destroyed by lyophilization.

The dose administered to a patient, in the context of the present invention should be sufficient to effect a beneficial therapeutic response in the patient over time, or to inhibit infection by a pathogen. The dose will be determined by the efficacy of the particular vector employed and the condition of the patient, as well as the body weight or surface area of the patient to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular vector, or transduced cell type in a particular patient.

In determining the effective amount of the vector to be administered in the treatment or prophylaxis of virally-mediated diseases such as AIDS, the physician evaluates circulating plasma levels, vector toxicities, progression of the disease, and the production of anti-vector antibodies. In general, the dose equivalent of a naked nucleic acid from a vector is from about 1 μg to 100 μg for a typical 70 kilogram patient, and doses of gene therapy vectors which include viral particle such as AAV or retroviral vectors are calculated to yield an equivalent amount of inhibitor nucleic acid.

In the practice of this invention, compositions can be administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically or intrathecally. The preferred method of administration will often be oral, rectal or intravenous, but the vectors can be applied in a suitable vehicle for the local and topical treatment of virally-mediated conditions. The vectors of this invention can supplement treatment of virally-mediated conditions by any known conventional therapy, including cytotoxic agents, nucleotide analogues and biologic response modifiers.

For administration, inhibitors and transduced cells of the present invention can be administered at a rate determined by the LD-50 of the inhibitor, vector, or transduced cell type, and the side-effects of the inhibitor, vector or cell type at various concentrations, as applied to the mass and overall health of the patient. Administration can be accomplished via single or divided doses.

Prior to infusion, blood samples are obtained and saved for analysis. Between 1 X 10^8 and 1 X 10^{12} transduced cells are infused intravenously over 60-200 minutes. Vital signs and oxygen saturation by pulse oximetry are closely monitored. Blood samples are obtained 5 minutes and 1 hour following infusion and saved for subsequent analysis. Leukopheresis, transduction and reinfusion are repeated every 2 to 3 months for a total of 4 to 6 treatments in a one year period. After the first treatment, infusions can be performed on a outpatient basis at the discretion of the clinician. If the reinfusion is given as an outpatient, the participant is monitored for at least 4, and preferably 8 hours following the therapy.

Transduced cells are prepared for reinfusion according to established methods. *See*, Abrahamsen *et al.* (1991) *J. Clin. Apheresis* 6:48-53; Carter *et al.* (1988) *J. Clin. Arpheresis* 4:113-117; Aebersold *et al.* (1988), *J. Immunol. Methods* 112: 1-7; Muul *et al.* (1987) *J. Immunol. Methods* 101:171-181 and Carter *et al.* (1987) *Transfusion* 27:362-365. After a period of about 2-4 weeks in culture, the cells should number between 1 X 10^8 and 1 X 10^{12}. In this regard, the growth characteristics of cells vary from patient to patient and from cell type to cell type. About 72 hours prior to reinfusion of the transduced cells, an aliquot is taken for analysis of phenotype, and percentage of cells expressing the therapeutic agent.

If a patient undergoing infusion of a vector or transduced cell develops fevers, chills, or muscle aches, he/she receives the appropriate dose of aspirin, ibuprofen or acetaminophen. Patients who experience reactions to the infusion such as fever, muscle aches, and chills are premedicated 30 minutes prior to the future infusions with either aspirin, acetaminophen, or diphenhydramine. Meperidine is used for more severe chills and muscle aches that do not quickly respond to antipyretics and antihistamines. Cell infusion is slowed or discontinued depending upon the severity of the reaction.

EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill will readily recognize a variety of noncritical parameters which could be changed or modified to yield essentially similar results.

Example 1: AAV Targeting

Ideally, a targetable gene therapy vector should have the following characteristics: 1) it should be simple to produce at an effective titer, 2) it should be stable so that storage and manipulation of the vector are not prohibitive issues, 3) it should bind specifically and deliver genes of interest to target cells, 4) It should be relatively void of binding to non-target cells, and 5) it should possess safety features which allow for effective and ethical use of the vector, ideally in a large number of individuals.

Toward these goals, mutations within the AAV capsid protein were identified which significantly reduce binding of the particle to the normal AAV receptor. In addition, evidence is presented in Example 2 demonstrating that a single-chain antibody was incorporated into an AAV vector particle as a cap fusion protein, and that these vectors target the particle to a new surface marker of interest (CD34). Based on these results and additional strategies outlined in this document, recombinant adeno-associated virus vectors (rAAV) can be genetically engineered to deliver genes of interest (*e.g.*, ribozymes) to specific target cell types and will contain all of the desirable features listed above. Described herein are three general strategies for accomplishing these goals in AAV vectors: 1) incorporation of targetable single-chain antibodies into vector particle capsid fusion proteins at specified regions of the protein (*i.e.*, N-terminus, C-terminus and location of binding mutation); 2) combinatorial library technology both for production of new targeting peptides and optimization of targetable vector assembly; and, 3) attaching targeting molecules of interest to the surface of pre-formed vector particles.

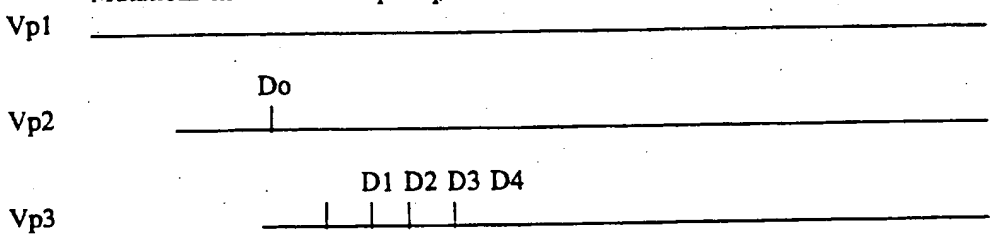
The basic strategy for creating a targetable rAAV vector was to first delete the normal binding region on the AAV capsid protein and then replace the region with a ligand capable of targeting the virus to a receptor other than AAV. The initial stages of this work were performed in the context of the WT virus, because it is easier to work with than recombinant virus. Subsequently, the

appropriate mutant capsid proteins were transferred to an rAAV helper plasmid used in standard rAAV packaging experiments.

Because the receptor binding region on AAV was previously unknown, a series of mutagenesis experiments were performed (*see*, below) to identify AAV receptor binding regions. The strategy for selecting the various sites for mutagenesis is indicated in Table 1. Two of the mutants made were binding deficient (Vp1hydro and D4). These vectors are used to construct a vector targeting $CD34^+$ cells with stem cell factor (SCF).

Table 1

Mutations in the AAV capsid proteins



Mutation	Position (amino acid)	Comments
Vp1	24-135	eliminates Vp1
Vp2	24-175	eliminates Vp1 and Vp2
Vp1 hydro	26-34	deletes proline rich region in Vp1
Do	192-197	5 amino acid deletion in Vp2*
D1	208-213	5 amino acid deletion in Vp3*
D2	217-222	5 amino acid deletion in Vp3*
D3	229-234	5 amino acid deletion in Vp3*
D4	239-244	5 amino acid deletion in Vp3*

*These mutations correspond to a region that has homology to the known binding site of the parvovirus B19

AAV Mutants. Oligonucleotide site-directed mutagenesis was performed by polymerase chain reaction (PCR). For this purpose, the Bst EII-Sna B1 fragment spanning nucleotides 1700 to 4496 in the AAV-2 genome (Srivatava, *et al.* (1983) *J. Virol.* 45: 555-564) was excised from pAV2, and site directed mutagenesis was performed on the purified fragment. Subsequently, the fragment was cloned back into pAV2, or alternatively into the AAV helper plasmid Ad8 (Samulski *et al.* (1989) *J. Virol.* 63: 3822-3828).

The **Vp1hydro** deletion is shown in bold underline text with flanking nucleic acids. The deletion corresponds to nucleic acids 2278-2304 as given in Srivatava, *et al.* (1983) *J. Virol.* 45: 555-564.

Vp1hydro: cagtggtggaagctc**aaacctggcccaccaccaccaaagcccg**cagagcggcataa.

The D_1 deletion is shown in bold underline text with flanking nucleic acids. The deletion corresponds to nucleic acids 2828-2834 as given in Srivatava, *et al.* (1983) *J. Virol.* 45: 555-564.

D1: gctacaggcagtggc**gcaccaatggcagaca**

The D_2 deletion is shown in bold underline text with flanking nucleic acids. The deletion corresponds to nucleic acids 2855-2868 as given in Srivatava, *et al.* (1983) *J. Virol.* 45: 555-564.

D2: ataacgagggc**gccgacggagtgggt**aattcctccggaa

The D_3 deletion is shown in bold underline text with flanking nucleic acids. The deletion corresponds to nucleic acids 2890-2905 as given in Srivatava, *et al.* (1983) *J. Virol.* 45: 555-564.

D3: attggcat**tgcgattccacatgg**atgggcga

The D_4 deletion is shown in bold underline text with flanking nucleic acids. The deletion corresponds to nucleic acids 2921-2935 as given in Srivatava, *et al.* (1983) *J. Virol.* 45: 555-564.

D4: cagagtc**atcaccaccagcacc**cgaacctgggccc

One of skill will readily appreciate that certain minor corrections have been reported to the sequence of AAV-2 as originally given by Srivatava, *et al.* (1983) *J. Virol.* 45: 555-564.

Particle formation assay: After generating the above mutants, the plasmid DNAs were transfected into adenovirus infected HeLa cells to produce packaged vector. Cell lysates were generated by freeze thaw and subsequently treated with DNase 1. The genome of intact particles were protected from DNase 1 digestion. Each sample was then digested with proteinase K and extracted with phenol/chloroform, and serial dilutions of the extracts were loaded onto a slot blot apparatus. Hybridization was performed using a labeled probe complementary to a region of AAV not contained within the deletions. Intact particle (virion) formation was evident in the WT control, and in the Vp1hydro and D4 mutants.

In one illustrative experiment, adenovirus infected cells were transfected with rAAV containing the b-gal gene plus wild-type, Vp1hydro or D1 mutant AAV helper plasmid and 72 hours later the cells were lysed by freeze/thaw, clarified by centrifugation, heated to 56°C for 45 min and then incubated with DNase 1 for 60 min at 37°C. Subsequently, the samples were treated with proteinase K, phenol/chloroform extracted, and bound to a solid matrix with a slot blot apparatus. Vector nucleic acids were hybridized to a 32-P labeled b-gal probe (the b-gal gene is present in each vector). Results showed that a Vp1hydro mutation with a stem cell factor insert had levels of particle formation similar to wild type AAV.

Infectivity assay:

Following particle formation, samples positive for particle formation (WT, Vp1hydro and D4) were normalized in terms of genome content and used to infect adenovirus infected HeLa cells. Cell lysates were then treated in a manner identical to the particle formation assay.

There was a significant reduction in the ability of Vp1hydro and D4 to replicate in HeLa cells relative to WT AAV. Because both of these mutations lead to intact particle formation as measured by nuclease resistance, the data suggests that the deletions result in reduced binding, rather than post-binding, events. This is particularly shown by the fact that transfection of the mutant DNAs in the particle formation assay gave a much stronger signal than the infectivity assay, even though transfection is usually much less efficient than infection. Decreased signals of Vp1hydro and D4 relative to the WT in the particle formation assay are expected since the mutations prevent subsequent rounds of infection after transfection. Conversely, after transfection the WT was able to infect untransfected cells and thereby amplify the signal that was observed.

Competition Binding Assay: the following competition binding assay was performed to demonstrate that the capsid mutations reduce the ability of the mutant virus to bind to host cells. 200 Cos cells were seeded onto a 24 well plate containing RPMI + 10% FBS in triplicate for each determination. After 24 hours incubation at 37°C the cells were transferred to 4°C and the indicated amounts of the various competitor virus was added in the presence of a fixed concentration of WT rAAV vector containing the b-gal gene. The cells were incubated for 60 min at 4°C and washed three times with cold RPMI and fresh RPMI + 10% FBS was added

followed by incubation at 37°C overnight. Subsequently, the cells were processed for b-gal staining by standard techniques. A decrease in the number of positive cells in the presence of increasing competitor is indicative of virus binding.

number of competitor virions per well	% positive cells			
	WT	WT(hi)*	Vp1hydro	Vp1hydro/SCF
0	35.1	26.0	31.5	33.9
10^7	23.0	22.8	36.6	33.0
10^8	14.6	25.0	28.3	25.9
10^9	10.3	29.0	26.6	24.9
10^{10}	8.5	28.3	24.0	ND

* WT AAV that has been denatured by heat inactivation at 100°C for 15 minutes

A small degree of binding to the normal AAV receptor is evident in the mutants; however, binding is substantially diminished compared to WT AAV as the competitor. Specifically, using WT AAV as the competitor reduces binding by a maximum of 75% while using the mutant as a competitor only reduces binding by 15%.

Targeting Analysis: The indicated cell lines (Table 2) were transduced with rAAV vectors containing capsid proteins AAV/Ad (WT AAV capsid), AAV/Ad vp1hydro (AAV binding mutant capsid), or AAV/Ad vp1hydro SCF (AAV binding mutant capsid with SCF insert). All vectors contained the b-gal gene as a reporter. As an additional positive control an adenovirus vector, Ad b-gal, was included.

Table 2

		blue cells
A.	Cos cells 5,000 cells per transduction	
	AAV/Ad	80
	AAV/Ad vp1hydro	0
	AAV/Ad vp1hydro SCF	0
	Control (normal cell lysate)	0
B.	HeLa cells 5,000 cells per transduction	
		blue cells
	AAV/Ad	41
	AAV/Ad vp1hydro	0
	AAV/Ad vp1 hydro SCF	0
	Ad b-gal	92
	Control (normal cell lysate)	0
C.	NCI 187 cells 5000 cells per transduction	
		blue cells
	AAV/Ad	62
	AAV/Ad vp1hydro	0
	AAV/Ad vp1hydro SCF	46
	Ad b-gal (MOI 10)	246
	Ad b-gal (MOI 30)	612
	Control (normal cell lysate)	0

Example 2: Targeted Delivery of Recombinant Adeno-associated Virus Vector (rAAV) Using Single Chain Antibody Against CD34

To achieve targeted delivery of rAAV *in vivo*, we engineered a chimeric AAV virion protein (VP) carrying coding sequences for the variable regions of a single chain antibody (scFv) against human CD34 molecules. Inclusion of CD34 single chain antibody-AAV capsid chimeric proteins in rAAV virions dramatically increased the infectivity of rAAV to $CD34^+$ KG-1 cells, a human myelogenous leukemia cell line that is normally resistant to rAAV transduction.

Hematopoietic stem cells are important targets for gene therapy. After the transduced stem cells differentiate, they reconstitute the hematopoietic system and can carry integrated transgenes into desired lineages of blood cells. Adenoviral vectors are good candidates for direct *in vivo* gene delivery to hematopoietic stem cells because they can transduce non-dividing cells (unlike retroviral vectors) and can mediate the integration of transgenes into host genomes (Fisher-Adams, G., *et al.*, *Blood* **88**:492 (1996)). However, for safety concerns, to transduce hematopoietic stem cells *in vivo*, it is desirable to develop AAV vectors that bind specifically to these cells.

In this Example, we incorporated the single chain fragment variable region (scFv) of a monoclonal antibody against human CD34 molecule, a marker for hematopoietic stem cells, (Civin, C. I., *et al.*, *J. Immunol.* **133**:157 (1984); Ogawa, M. *Blood* **81**:2844 (1993)), into the AAV virion via a VP-scFv chimeric protein.

A. Cell culture and DNA transfection.

HeLa cells were grown in Delbecco's Modified Eagle Media (DMEM) supplemented with antibiotics and 10% fetal bovine serum. KG-1 (ATCC CCL-246), a human acute myelogenous leukemia cell line that expresses CD34 molecules, (Koeffler, H.P. and Golde, D.W., *Science* **200**:1153 (1978); Simmons, D.L., *et al.*, *J. Immunol.* **148**:267 (1992); and Civin, C.I., *et al.*, (1984)), was cultured in Iscove's Modified Eagle Medium supplemented with 20% fetal bovine serum. The anti-CD34 hybridoma My-10 (ATCC HB8483) was cultured in RPMI medium in the presence of 10% fetal bovine serum. All cells were maintained at 37°C in a 5.0% CO_2 atmosphere. DNA transfections of HeLa cells were performed by calcium phosphate transfection one day after plating onto culture dishes (*see*, Ausubel F.M., *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Greene Publishing Associates, Brooklyn, N.Y. (1987)).

B. Cloning and Expression of scFv From My-10 Hybridoma.

PCR amplification with degenerate primers from an Ig-primer kit (Novagen) was utilized to clone the variable heavy and light chain sequences of the My-10 hybridoma genome. Briefly, total RNA was isolated from My-10 cells and the mRNA reverse transcribed into cDNA with AMV reverse transcriptase. PCR amplification of the heavy chain and light chain sequences was carried out with different combinations of degenerate primers provided for in the kit. The resulting PCR fragments (about 450 base pairs) were subcloned into TA cloning vectors (Invitrogen) and sequenced (Sequenase, U.S.B). Three out of six of the light and heavy chain primer pairs produced PCR products upon amplification; however, only primer pairs MulgkV_L5'-G and MulgkV_L3'-1, and MulgV_H5'-C and MulgV_H3'-2 of the Ig-Primer kit generated DNA fragments which coded for the N-terminal and C-terminal consensus sequence of V_L and V_H, respectively. The sequences of multiple clones were compared and representative clones were chosen and ligated into pBlueScript vectors (Strategene) carrying a linker sequence, $(GGGGS)_3$.

The resulting scFv with a structure of V_H-$(GGGGS)_3$-V_L comprised an N-terminal heavy chain region of 116 amino acids followed by the linker sequence and a C-terminal light chain region of 112 amino acids.

The cloned scFv gene was inserted into a pTrc/his vector (Invitrogen) for bacterial expression of the scFv with a polyhistidine tag. The histidine tagged scFv protein was purified using a nickel column under denaturing conditions. Renaturation was performed by dialyzing the purified proteins against 10 mM Tris-HCl, pH 7.8, and 0.1% Triton X-100. Any undissolved proteins were removed before further use and the solubilized protein was identified as scFv-his by western blot analysis using an anti-his C-terminal antibody (Invitrogen).

C. Expression of ScFv-AAV capsid chimeric proteins in vitro and in vivo.

Standard procedures were followed for plasmid construction, growth and purification (*see*, Ausubel, *supra*). The capsid genes for VP1, VP2, and VP3 were amplified from the pAV2 plasmid, which contains the full length AAV-2 genome (Laughlin, C.A., *et al.*, *Gene* 23:65 (1983)). The 3' primers corresponded to the end of the AAV capsid gene open reading frame and the 5' primers corresponded to the start codon of individual capsid genes, i.e. position 2203-2229 for VP1, 2617-2640 for VP2, and 2810-2833 for VP3. The 3' and 5' primers were synthesized so that Sal I and Xba I sites would be incorporated into the VP sequences for ligation into vectors. The amplified sequences with the incorporated restriction sites were subcloned into pCDNA3 expression vectors (Invitrogen) via Xho I and Xba I sites to generate pVP1, pVP2, and pVP3 plasmids.

The above cloned anti-CD34 scFv sequence was ligated to the 5' end of the VP1, VP2, and VP3 sequences using Hind III and Not I sites to generate the plasmids pVP1-scFv, pVP2-scFv, and pVP3-scFv.

The expression of the various AAV virion proteins and the chimeric proteins were verified *in vitro* using a TNT transcription and translation kit (Promega). We detected the expression of AAV VP1, VP2, and VP3 proteins as well as VP1-scFv, VP2-scFv, and VP3-scFv.

For *in vivo* expression, 2 μg of the plasmids were transfected into 2 x 10^5 HeLa cells in 60 mm dishes by calcium phosphate transfection. Forty eight hours later, the cells were collected and cell lysates prepared by adding 30 μL lysis

buffer (10 mM Tris, pH 7.8, 2 mM PMSF, 5 mM $MgCl_2$, 0.3 M NaCl, and a proteinase inhibitor cocktail from Boehringer Mannheim) and boiling for 15 min with 10 μL 3X electrophoresis loading buffer. The presence of intracellular chimeric proteins was verified by western blot analysis using guinea pig anti-AAV2 antibody (NIH) and HRP-conjugated goat anti-guinea pig antibody. The ECL system (Amersham) was used to detect positive signals.

From the western blot analysis, it was clear that the VP2-scFv and VP3-scFv could be detected after transient transfection. Due to non-specific binding of the detection antibody to cellular proteins, it was not clear whether VP3-scFv was expressed.

D. Production of rAAV Vector and rAAV Transduction.

Initial attempts to make a packaging a recombinant AAV (rAAV) genome containing one or two of the chimeric virion proteins failed to produce any intact viral particles. To overcome this obstacle, we included wild type AAV capsid proteins into the packaging process. We employed a triple plasmid DNA co-transfection strategy, namely co-infecting cells with (1) pAV/Ad, (2) pAAVgal conjugated to polylysine coupled adenovirus, and (3) the individual pVP-scFv chimeric protein-containing plasmid.

To produce the rAAV particles, about 1 $x10^7$ HeLa cells in T162 flasks were transfected with 10 μg of plasmid pAV/Ad, which has the entire coding sequence for AAV replication and capsid genes (Samulski, R.J., *et al., J. Virol.* 63:3822 (1989)), and 10 μg plasmid pAVgal, an AAV vector plasmid containing a bacterial *lacZ* gene under the control of the cytomegalovirus (CMV) early promoter, conjugated to 100 μL polylysine coupled adenovirus (M.O.I. about 10) (Mamounas, M., *et al., Gene Ther.* 2:429 (1995)). After 48-72 hr, about 80% of cells showed maximum cytopathic effect (CPE) and the cells were harvested and resuspended into a small volume of culture medium.

The rAAV particles were extracted from HeLa cells after three freezing and thawing cycles. The crude lysates were spun in a microcentrifuge for 10 min at maximum speed. The rAAV particles were purified by CsCl centrifugation. 30-40 T162 flasks of rAAV-containing lysates were combined and CsCl was added to the lysate to a final density of 1.4 g/ml. After centrifugation at 35,000 rpm, cell debris and the majority of the adenoviruses were removed and a

second centrifugation was performed. The contents of the centrifuge tube were collected in 1 mL fractions. Each fraction was tested for the presence of intact rAAV particles by slot blot analysis using a *lacZ* gene probe. The fractions with the highest rAAV particle titer were pooled and dialyzed against DMEM medium for 2 hr at 40°C with one change of dialysis medium.

For transduction with rAAV particles, about 2 x 10^4 KG-1 or HeLa cells were plated in 24-well plates one day prior to transduction. Appropriate amounts of rAAV were added to the media, and the particles and cells were incubated overnight. After the incubation period, the cells were washed and the expression of the *lacZ* gene was detected by X-gal staining.

To generate AAV vectors (vAAV) which contained the VP-scFv chimeric proteins; 1 x 10^7 HeLa cells in T162 flasks were transfected with, in addition to the plasmids pAV/Ad and pAVgal, 10μg of pVP1-scFv, pVP2-scFv, and pVP3-scFv.

Screening of vVP1-scFvgal, vVP2-scFvgal, and vVp3-scFvgal (the vAAV purified from HeLa cells) for their infectivity on HeLa cells and $CD34^+$ KG-1 cells demonstrated that the inclusion of pVP2-scFv greatly increased the infectivity of vAAV on KG-1 cells, which normally is resistant to rAAV infection under the conditions we used. Consequently, we focused our efforts on vVP2-scFv. We produced large quantities of vVP2-scFvgal and purified it by CsCl gradient centrifugation. The differential infectivity of vVP2-scFvgal and vAAVgal on KG-1 cells is summarized in Table 3. Although similar transduction efficiency was observed in HeLa cells, vVP2-scFvgal transduced KG-1 cells at a titer about 100 infectious particles/ml, while vAAVgal failed to transduce at all. The increased transduction by vVP2-scFvgal on KG-1 cells has been observed using crude lysates as well.

Table 3: Infectivity of CsCl Purified vAAVgal on HeLa and KG-1 Cells

Viruses	Infection (Titer/mL)	
	HeLa Cells	KG-1 cells ($CD34^+$)
vAAVgal	1.3×10^6	0
vVP2-scFvgal	8.8×10^5	90

E. Flow Cytometry and Binding of scFv to CD34 Molecules.

For the binding of histidine tagged scFv from transformed *E. coli* to CD34 molecules, about 1×10^5 of KG-1 cells were incubated with or without 1 μg of scFv protein or control proteins at 40°C for 30 min. About 0.05 μg of phycoerythrin conjugated ICH3 antibody (PE-ICH3, 1mg/ml, Caltag Lab., CA) was added and incubated for an additional 30 min.. After washing with PBS, the stained cells were subject to flow cytometry analysis using FACS Vantage (Becton Dickinson).

To analyze the binding of CsCl-purified vAAVgal carrying VP2-scFv chimeric proteins to CD34 molecules, about 1×10^5 of KG-1 cells were incubated with or without 1×10^{10} particles of vVP2-scFvgal or 1×10^{10} particles of vAAVgal at 40°C for 30 min. About 0.05 μg of PE-ICH3 was added and incubated for an additional 30 min. After washing with PBS, the stained cells were subject to flow cytometry analysis using FACS Vantage (Becton Dickinson).

We next studied the specificity of vVP2-scFvgal for CD34 in transduced KG-1 cells. Because of difficulties in conjugating My-10 antibodies with commercially available fluorescent reagents, it proved to be difficult to investigate the binding of purified histidine tagged scFv to CD34 molecules on the surface of KG-1 cells. As a result, we developed a competitive binding assay based on the observation that My-10 monoclonal antibodies blocked the binding of ICH3 monoclonal antibodies to CD34 molecules (Gaudernack, G. and Egeland, T., LEUCOCYTE TYPING V; WHITE CELL DIFFERENTIATION ANTIGENS, PROCEEDINGS OF THE FIFTH INTERNATIONAL WORKSHOP AND CONFERENCE, VOL. I., Schlossman S. F. *et al.* (eds.), Boston, MA, November 3-7, 1993, Oxford University Press, New York (1995)).

We first determined that 0.05 μg PE-ICH3 was the minimum amount required for detection of 1×10^5 KG-1 cells. In the competitive binding assay, we

preincubated KG-1 cells with 1 μg of histidine tagged scFv or control protein for 30 minutes before the addition of PE-ICH3 antibody. The addition of dialysis medium, control IgG_1 or histidine tagged lacZ protein did not interfere with the binding of ICH3 to KG-1 cells, however, the presence of purified scFv reduced the binding of ICH3 to CD34 by more than 90%. This indicates that the scFv we purified bound to CD34 molecules on the KG-1 cell surface.

The preincubation of 1 x 10^{10} particles of vVP2-scFv reduced the binding of ICH3 antibody to CD34 molecules by almost 90%, while vAAVgal reduced binding of ICH3 by about 35%. This was most likely due to non-specific inhibition of ICH3.

By increasing the amount of PE-ICH3 added to the cells, the inhibition by vVP2-scFvgal was no longer observed. This demonstrated that the VP2-scFv chimeric protein expressed on the vVP2-scFvgal surface mediated the binding of vVP2-scFvgal to CD34 molecules on KG-1 cell surface. This finding showed that specific targeting of AAV vectors into KG-1 cells as well as other $CD43^+$ cells can be achieved.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A recombinant targeted viral vector comprising a nucleic acid and a viral particle, which particle comprises a targeting ligand and has reduced specificity for a cellular receptor as compared to a corresponding wild-type virus.

2. The recombinant targeted viral vector of claim 1, wherein the vector comprises a viral particle selected from the group consisting of AAVs, Ads, parvoviruses, herpesviruses and retroviruses.

3. The recombinant targeted viral vector of claim 1, wherein the targeting ligand is selected from the group of ligands consisting of an antibody, an antibody binding ligand, a cellular receptor ligand, a polypeptide, a C4 peptide, polylysine and streptavidin.

4. The recombinant targeted viral vector of claim 1, wherein the targeting ligand is encoded by the nucleic acid.

5. The recombinant targeted viral vector of claim 1, wherein the vector further comprises an expression cassette.

6. The recombinant targeted viral vector of claim 1, wherein the vector further comprises an expression cassette, which expression cassette encodes a ribozyme.

7. A method of transducing a cell with a target nucleic acid, comprising contacting the cell with the vector of claim 5.

8. The method of claim 7, wherein the vector comprises a streptavidin moiety on the surface of the vector, wherein the method further comprises binding a biotinylated antibody to the vector, which biotinylated antibody binds to a molecule on the surface of the cell.

9. The vector of claim 8, wherein the vector particle is derived from a virus selected from the group consisting of Ads, parvoviruses, herpesviruses and retroviruses.

10. The vector of claim 1, wherein the targeting ligand is an antibody which specifically binds to a human CD34 protein.

11. A nucleic acid which encodes the recombinant viral vector of claim 1.

12. The nucleic acid of claim 11, wherein the nucleic acid encodes an AAV ITR.

13. The nucleic acid of claim 12, wherein the nucleic acid further comprises a nucleic acid subsequence selected from the group consisting of Vp1 hydro and D4.

14. A targeted adenovirus nucleic acid encoding a target ligand in the L5 region of an adenoviral genome.

15. The nucleic acid of claim 14, wherein the target ligand is selected from the group of ligands consisting of an antibody, an antibody binding ligand, a cellular receptor ligand, a polypeptide, the C4 peptide and streptavidin.

16. The nucleic acid of claim 14, wherein an adenovirus encoded by the targetable nucleic acid has reduced specificity for an adenovirus receptor protein.

17. The nucleic acid of claim 14, wherein the nucleic acid is packaged in a viral particle with components encoded by the nucleic acid.

18. A targetable AAV vector comprising a nucleic acid and a capsid which packages the nucleic acid, which capsid has reduced specificity for an AAV cellular receptor.

19. The vector of claim 18, wherein the capsid comprises a deletion selected from the group of deletions consisting of a deletion in Vp1, and a deletion in Vp3.

20. The vector of claim 19, wherein the deletion is selected from the group of deletions consisting of a deletion in Vp3 comprising a deletion of amino acids 239-244 from Vp3, and a deletion in Vp1 comprising a deletion in the proline rich region of Vp1.

21. The vector of claim 20, wherein the deletion is selected from the group of deletions consisting of a deletion of amino acids 239-244 in Vp3, and a deletion of amino acids 26-34 in Vp1.

22. The vector of claim 18, wherein the vector comprises a fusion cap protein comprising a targeting ligand domain and a cap protein domain, which cap protein domain has reduced specificity for the AAV cellular receptor as compared to a wild-type cap protein, which targeting ligand binds to a target cell.

23. The vector of claim 22, wherein the targeting ligand is expressed in a region of the capsid corresponding to a wild type AAV-2 selected from the group of regions consisting of Vp1, and Vp3.

24. The vector of claim 23, wherein the targeting ligand is expressed in a region of the capsid corresponding to a wild type AAV-2 selected from the group of regions consisting of amino acids 26-34 of Vp1 and 239-244 of Vp3.

25. A nucleic acid which encodes the vector of claim 18.

26. A method of transducing a cell with a target nucleic acid comprising contacting a target cell with the vector of claim 18.

27. The method of claim 26, wherein the capsid comprises a targeting ligand which binds a cell surface molecule, wherein the method further comprises contacting the capsid with the cell targeting ligand.

28. The method of claim 26, wherein the cell is contacted *in vitro*.

29. The method of claim 27, wherein the cell targeting ligand is a biotinylated protein and the capsid comprises a streptavidin moiety.

30. The method of claim 29, wherein the streptavidin moiety is integrated into the viral capsid by cloning the streptavidin moiety into a nucleic acid encoding a capsid protein and translating the nucleic acid.

31. A non-enveloped viral vector selected from the group of non-enveloped vectors consisting of Ad, and AAV, which vector comprises a target nucleic acid and a recombinant capsid, which recombinant capsid has reduced specificity for a cellular receptor as compared to a wild-type capsid, wherein the recombinant capsid further comprises a targeting ligand.

32. The non-enveloped viral vector of claim 31, wherein the targeting ligand is an antibody which binds to a cell receptor selected from the group consisting of CD34 and CD4.

33. A non-enveloped viral vector selected from the group of non-enveloped vectors consisting of Ad, and AAV, which vector comprises a target nucleic acid and a capsid, wherein the capsid is bound to a biotinylated antibody, wherein the antibody inhibits entry of the virus into a cell through a cellular receptor which binds to a wild type virus corresponding to the viral vector.

34. The non-enveloped viral vector of claim 33, wherein the antibody binds to a viral recognition site on the capsid.

35. The non-enveloped viral vector of claim 33, wherein the composition further comprises a streptavidin moiety and a second antibody, which second antibody specifically binds to a molecule on the surface of a cell.

36. A method of transducing a cell with a target nucleic acid, comprising contacting the cell with the non-enveloped viral vector of claim 33.

37. The method of claim 36, further comprising contacting the viral vector with a streptavidin moiety and a second antibody, which second antibody specifically binds to a molecule on the surface of a cell, and which second antibody is biotinylated.

38. A targetable vector library comprising a plurality of recombinant targeted viral vectors having reduced specificity for a cellular receptor as compared to a corresponding wild-type virus, wherein each of the targeted viral vectors comprises a vector nucleic acid encoding a viral surface protein-targeting ligand fusion protein.

39. The targetable vector library of claim 38, wherein the recombinant targeted vectors are derived from AAV and the viral surface protein is a cap protein.

40. The targetable vector library of claim 38, wherein the targeting ligand binds to a cell receptor selected from the group consisting of $CD4^+$ and $CD34^+$.

41. The targetable vector library of claim 38, further comprising a recombinant cell which does not comprise a functional AAV receptor.

42. A method of selecting a targeted vector comprising the steps of:

providing a targeting nucleic acid encoding a targeting peptide, which peptide binds to a target cell;

randomly cloning the targeting nucleic acid into a vector nucleic acid comprising a mutant surface protein subsequence which subsequence encodes a viral surface protein, which protein binds to a viral receptor with reduced affinity compared to a corresponding wild type viral surface protein, thereby providing a library of random insertions of the targeting nucleic acid into the mutant surface protein subsequence; and,

selecting the library of random insertions for a vector that infects a target cell and binds the cell with increased affinity as compared to a corresponding wild type virus, thereby selecting the targeted vector.

43. The method of claim 42, wherein cloning the targeting nucleic acid into the vector nucleic acid further comprises the steps of:

randomly cloning the targeting nucleic acid into a plasmid comprising the mutant surface protein subsequence, thereby producing a targeting-surface protein fusion nucleic acid;

cleaving the plasmid with a restriction enzyme to release the fusion nucleic acid, thereby producing a released fusion nucleic acid; and,

cloning the released fusion nucleic acid into the vector nucleic acid.

44. The method of claim 43, wherein the vector nucleic acid encodes viral particle components.

45. The method of claim 42, wherein the targeted vector is an AAV vector.

46. The method of claim 45, wherein the step of selecting the library comprises

packaging AAV vectors in adenovirus infected cells, thereby providing packaged AAV vectors;

infecting target cells with the packaged AAV vectors, thereby providing infected target cells; and,

subjecting the infected target cells to a mutagen.

47. The method of claim 42, further comprising the steps of PCR amplifying the vector nucleic acid, thereby providing an amplified vector nucleic acid, and sequencing the amplified vector nucleic acid.

48. The method of claim 42, further comprising the steps of providing a random phage display library of potential targeting peptides; and,

selecting a member of the random phage display library which binds to a target cell, which member comprises a nucleic acid encoding a targeting peptide, thereby providing a targeting nucleic acid encoding a peptide which binds to a target cell.

49. A targeted vector isolated by the method of claim 42.

50. An AAV vector comprising a polypeptide domain which specifically binds to a cell receptor selected from the group consisting of CD4 and CD34.

51. The AAV vector of claim 50, wherein the polypeptide is a single chain antibody sFv fusion protein.

52. A nucleic acid encoding the vector of claim 50.

53. A cell comprising the nucleic acid of claim 52.

54. An isolated viral vector comprising a targeting ligand which binds to a cell surface protein, wherein the viral vector is made by the steps of:

isolating a bacteriophage particle which specifically binds to a cell comprising the cell surface protein from a random phage display library, thereby providing an identified bacteriophage particle;

subcloning a subsequence derived from the identified bacteriophage particle corresponding to the targeting ligand into a vector nucleic acid; and,

expressing the vector nucleic acid in a cell, thereby making a targeting protein comprising the targeting ligand, which targeting protein is packaged into the vector.

55. The vector of claim 54, wherein the vector is an AAV vector.

56. The vector of claim 54, wherein the vector binds to a cell surface protein selected from the group consisting of CD4 and CD34.

57. A polypeptide which binds to a CD4 cell surface protein comprising a peptide subsequence selected from the group consisting of GAVQPRGATSKLYLLRMTDK, MGEKLHRVHIRTNTPSVYSR, LEPRVAQRGQMVKFTYMRLP, HAWWKPWGWSIEALAPTAGP, and, conservative modifications thereof.

58. A first nucleic acid which hybridizes under stringent conditions to nucleic acid selected from the group consisting of: a coding nucleic acid which encodes a polypeptide of claim 57, and a complementary nucleic acid which is complementary to a nucleic acid which encodes a polypeptide of claim 57.

59. The nucleic acid of claim 58, wherein the nucleic acid further comprises a sequence encoding a viral capsid protein.

60. The nucleic acid of claim 58, wherein the nucleic acid is present in a viral particle, which particle comprises a targeting ligand and has reduced specificity for a cellular receptor as compared to a corresponding wild-type virus, wherein the targeting ligand comprises a polypeptide which binds to a CD4 cell surface protein comprising a peptide subsequence selected from the group consisting of GAVQPRGATSKLYLLRMTDK, MGEKLHRVHIRTNTPSVYSR, LEPRVAQRGQMVKFTYMRLP, HAWWKPWGWSIEALAPTAGP, and, conservative modifications thereof.

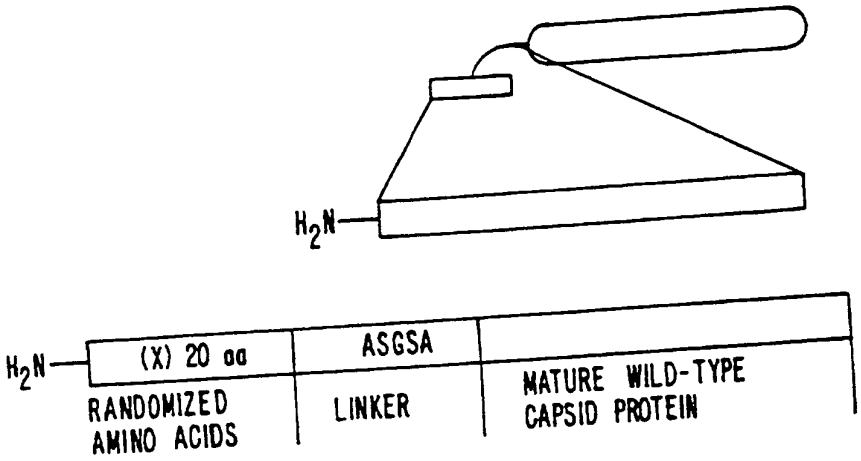

H_2N
H_2N
(X) 20 aa
RANDOMIZED AMINO ACIDS
ASGSA
LINKER
MATURE WILD-TYPE CAPSID PROTEIN

FIG. 1.

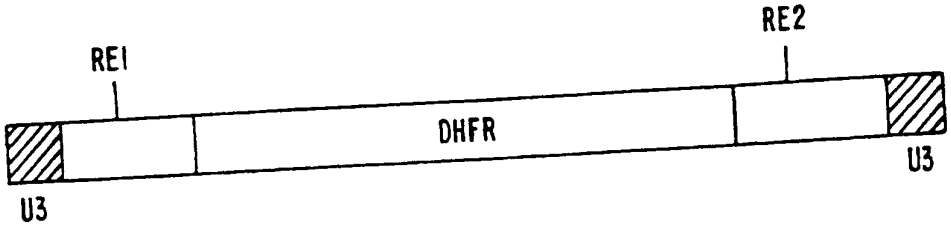

RE1
RE2
DHFR
U3
U3

FIG. 5.

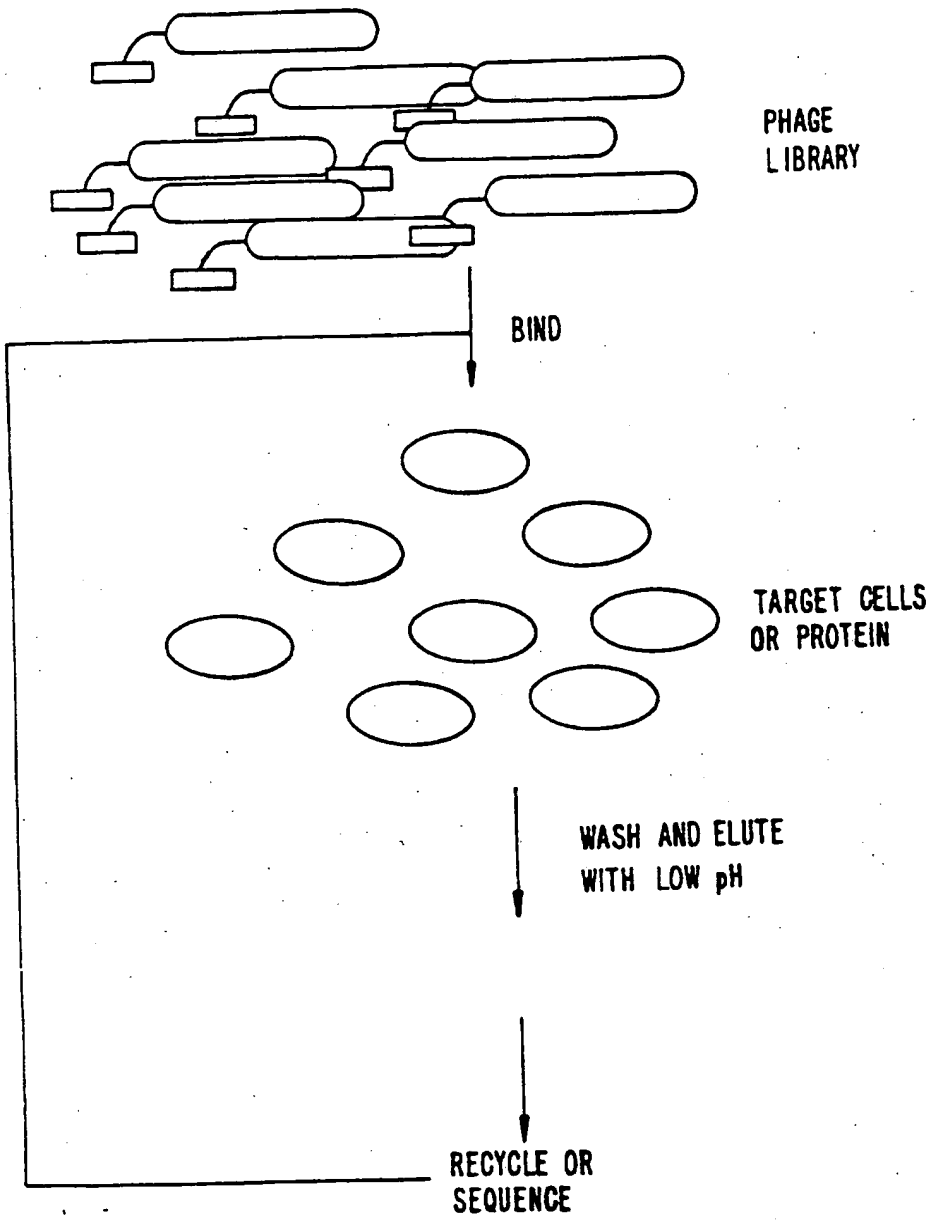

PHAGE
LIBRARY
BIND
TARGET CELLS
OR PROTEIN
WASH AND ELUTE
WITH LOW pH
RECYCLE OR
SEQUENCE

FIG. 2.

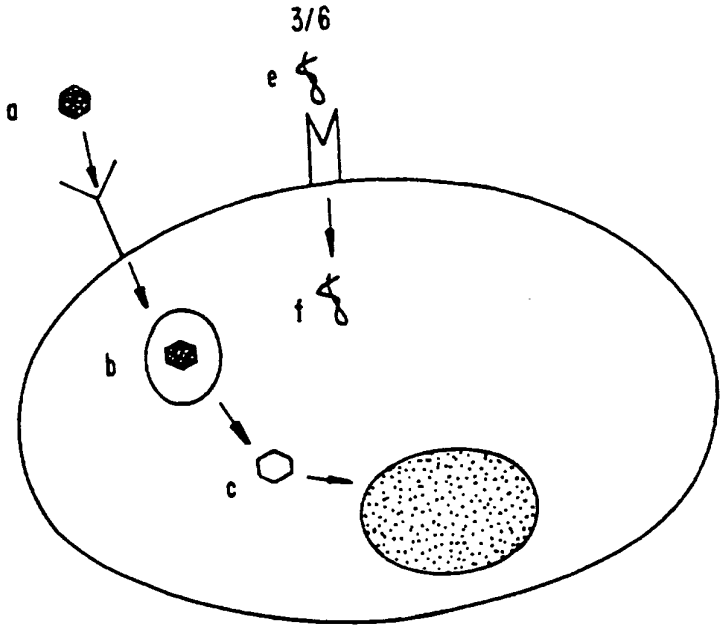


FIG. 3A.

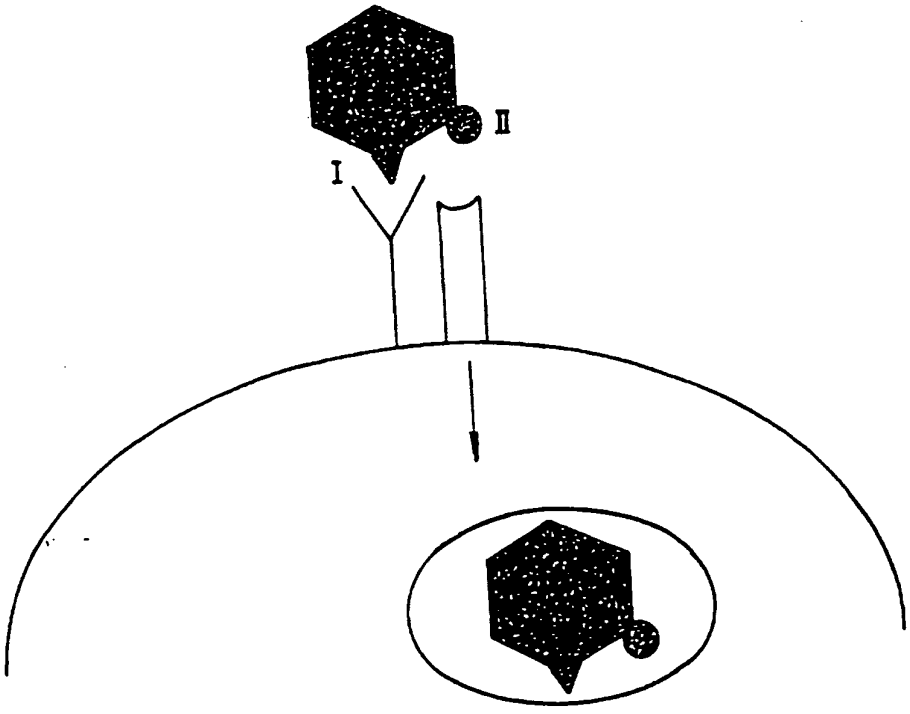


FIG. 3B.

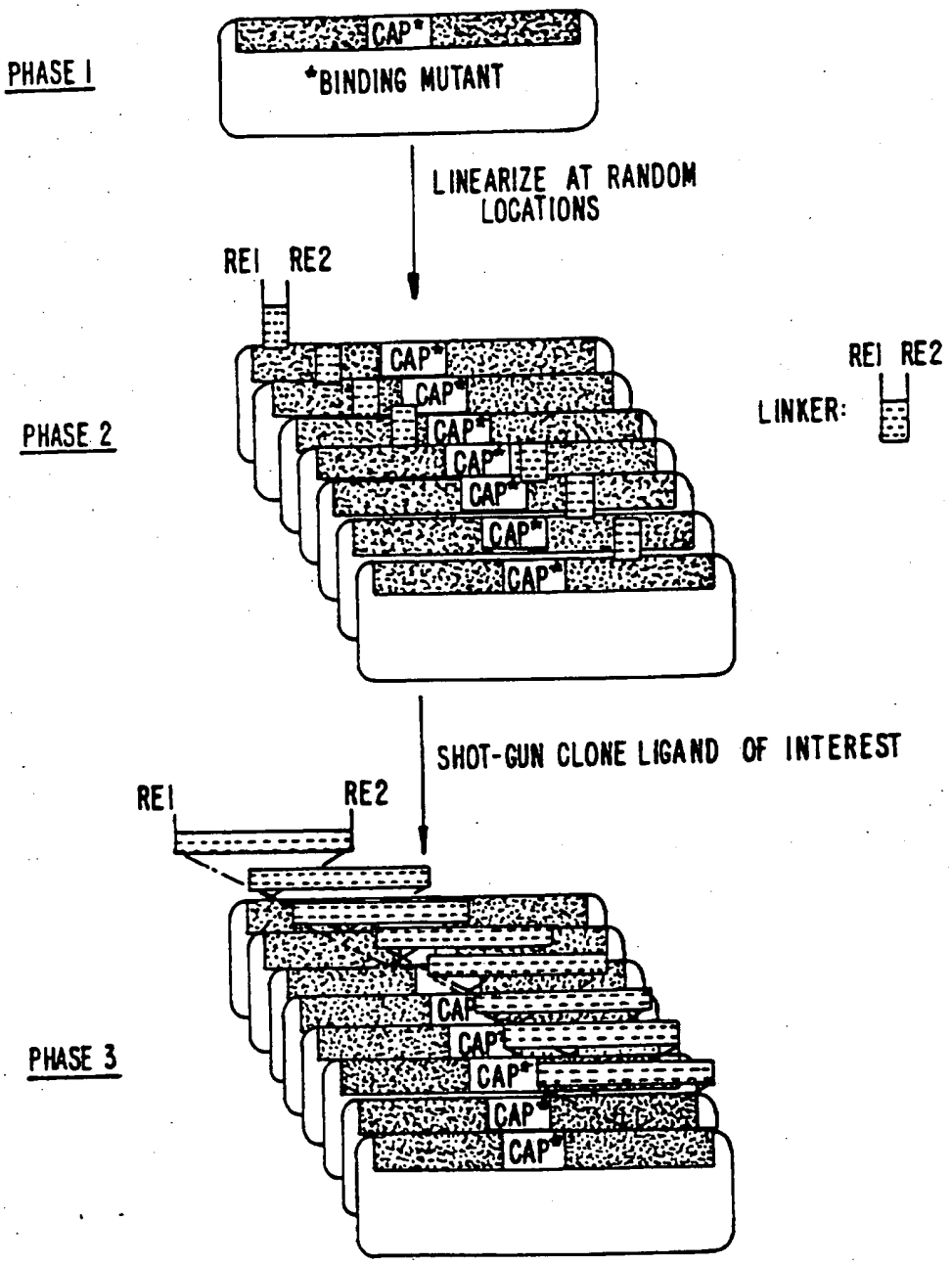

PHASE 1
CAP*
*BINDING MUTANT
LINEARIZE AT RANDOM LOCATIONS
RE1 RE2
PHASE 2
CAP*
RE1 RE2
LINKER:
SHOT-GUN CLONE LIGAND OF INTEREST
RE1
RE2
PHASE 3

FIG. 4-1.

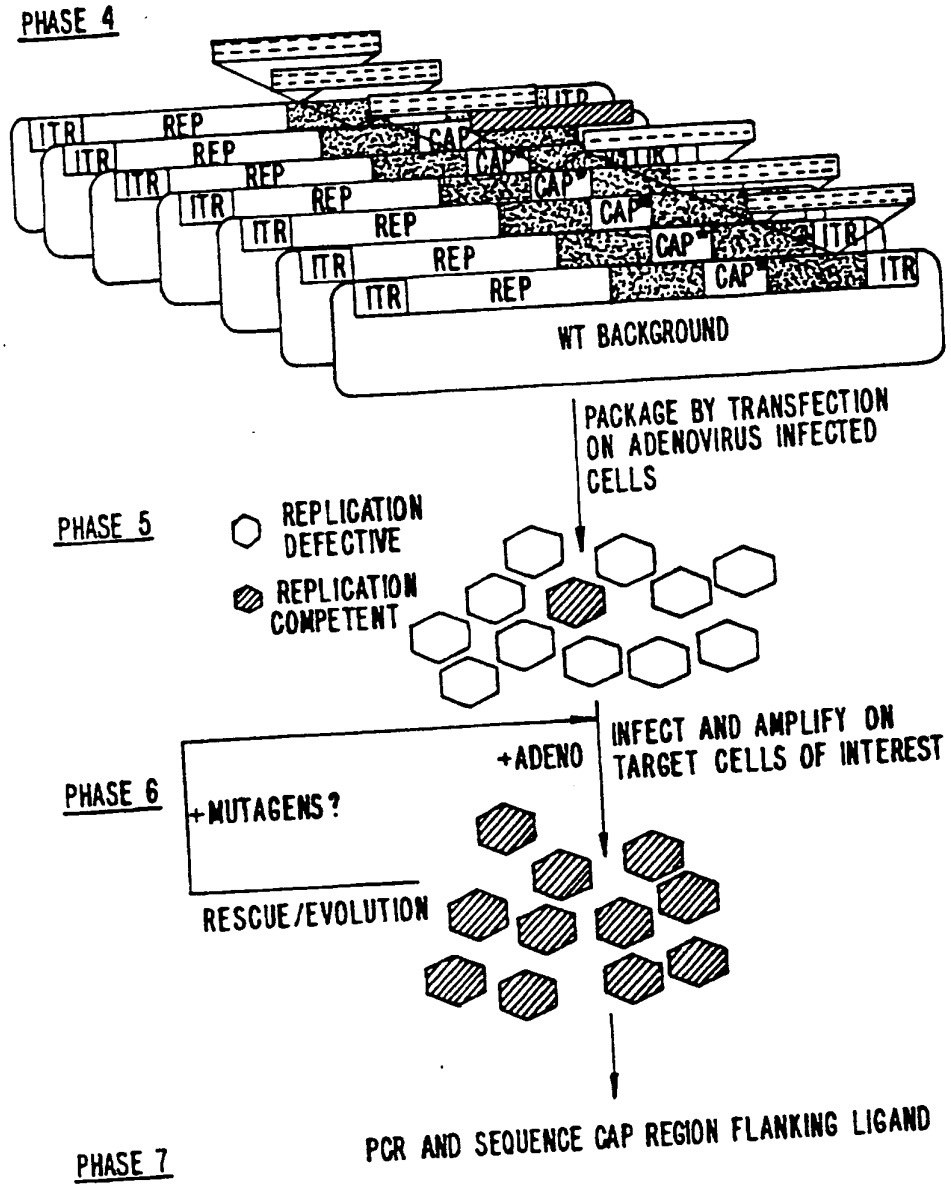
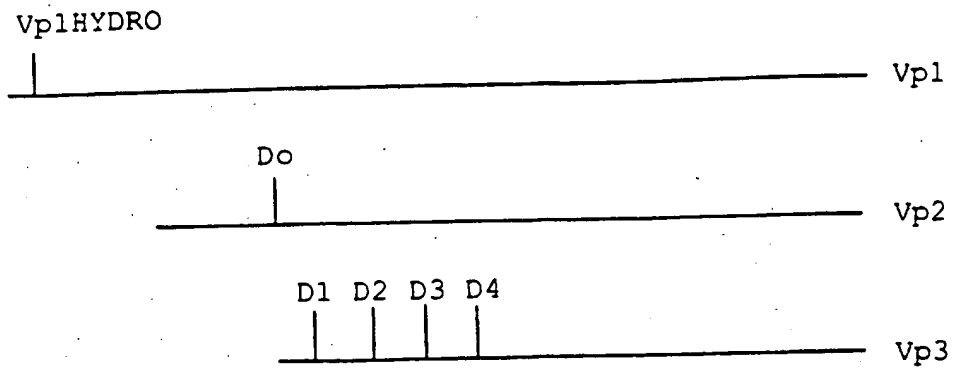

PHASE 4
ITR
REP
CAP
WT BACKGROUND
PACKAGE BY TRANSFECTION ON ADENOVIRUS INFECTED CELLS
PHASE 5
REPLICATION DEFECTIVE
REPLICATION COMPETENT
INFECT AND AMPLIFY ON TARGET CELLS OF INTEREST
PHASE 6
+ADENO
+MUTAGENS?
RESCUE/EVOLUTION
PHASE 7
PCR AND SEQUENCE CAP REGION FLANKING LIGAND

FIG. 4-2.



MUTATION	POSITION (AMINO ACID)	COMMENTS
Vp1	24-135	ELIMINATES Vp1
Vp2	24-175	ELIMINATES Vp1 AND Vp2
Vp1HYDRO	26-34	DELETES PROLINE RICH REGION IN Vp1
D0	192-197	5 AMINO ACID DELETION IN Vp2*
D1	208-213	5 AMINO ACID DELETION IN Vp3*
D2	217-222	5 AMINO ACID DELETION IN Vp3*
D3	229-234	5 AMINO ACID DELETION IN Vp3*
D4	239-244	5 AMINO ACID DELETION IN Vp3*

* THESE MUTATIONS CORRESPOND TO A REGION THAT HAS HOMOLOGY TO THE KNOWN BINDING SITE OF THE PARVOVIRUS B19

NOTE: Vp1, Vp2, D0, D1, D2, and D3 DID NOT FORM PARTICLES.

Vp1HYDRO, AND D4 FORMED PARTICLES AND WERE SHOWN TO HAVE MUTATIONS THAT DISRUPT BINDING

FIG. 6.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/06590

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/186.1, 187.1, 188.1, 192.1; 435/69.3, 69.6, 69.7, 172.3, 320.1; 536/23.4, 23.53, 23.72.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	SOMIA et al. Generation of targeted retroviral vectors by using single-chain variable fragment: An approach to in vivo gene delivery. Proc. Natl. Acad. Sci. USA. August 1995. Vol. 92, pages 7570-7574, see entire document	1-5, 7, 11, 49 ---------- 6, 8-10, 12-37, 50-56, 58-60
X -- Y	COSSET et al. Retroviral retargeting by envelopes expressing an N-terminal binding domain. J. Virology. October 1995. Vol. 69, No. 10, pages 6314-6322. See entire document.	1-5, 7, 11, 49 ---------- 6, 8-10, 12-37 50-56, 58-60

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
20 JUNE 1997

Date of mailing of the international search report
25 JUL 1997

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/06590

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO 95/23846 A (DORNBURG, R. C.) 08 September 1995, see entire document.	1-5, 7, 11, 49 --- 6, 8-10, 12-37, 50-56, 58-60
X -- Y	SCHWARZENBERGER et al. Targeted Gene transfer to human hematopoietic progenitor cell lines through the c-kit receptor. Blood. 15 January 1996. Vol. 87, No. 2, pages 472-478. See Abstract and page 472, first paragraph.	1-5, 7, 11, 49 --- 6, 8-10, 12-37, 50-56, 58-60
X -- Y	WO 93/09221 A (LINDHOLM et al.) 13 May 1993, see entire document.	1-5, 7, 11, 49 --- 6, 8-10, 12-37, 50-56, 58-60
Y	WONG et al. Primary human CD34+ peripheral blood stem cells (PBSCs) are efficient targets for adeno-associated virus vector-mediated gene transfer: Prospects for anti-AIDS genetic intervention. Blood. 1994. Vol. 84, No. 10, Suppl. 1, page 743A. See entire document.	1-37, 49-56, 58-60
Y	MILLER et al. Targeted vectors for gene therapy. The FASEB Journal. February 1995. Vol. 9, No. 2, pages 190-199, see entire document.	1-37, 49-56, 58-60
Y	US 4,797,368 A (CARTER et al.) 10 January 1989, see Abstract.	1-37, 49-56, 58-60
Y	US 5,474,935 A (CHATTERJEE et al.) 12 December 1995, see entire document.	1-37, 49-56, 58-60
Y	US 5,478,745 A (SAMULSKI et al.) 26 December 1995, see entire document.	1-37, 49-56, 58-60
Y, P	DOUGLAS et al. Targeted gene delivery by tropism-modified adenoviral vectors. Biotechnology. November 1996. Vol. 14, No. 11, pages 1574-1578. See entire document.	1-37, 49-56, 58-60
Y, P	WICKHAM et al. Targeted adenovirus gene transfer to endothelial and smooth muscle cells by using bispecific antibodies. J. Virology. October 1996. Vol. 70, No. 10, page 6831-6838. See entire document.	1-37, 49-56, 58-60
Y, P	WALTHER et al. Targeted vectors for gene therapy of cancer and retroviral infections. Molecular Biotechnology. December 1996. Vol. 6, No. 3, pages 267-286. See entire document.	1-37, 49-56, 58-60

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/06590

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-37, 49-56 and 58-60

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/06590

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 39/12, 39/21, 39/23, 39/235, 39/245, 39,395, 39/42, 39/44; C12N 15/12, 15/35, 15/38, 15/49, 15/62; C07H 21/04.

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/186.1, 187.1, 188.1, 192.1; 435/69.3, 69.6, 69.7, 172.3, 320.1; 536/23.4, 23.53, 23.72.

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-37, 49-56 and 58-60, drawn to targeted vectors and methods of transducing cells.
Group II, claim(s) 38-41, drawn to targeted vector libraries.
Group III, claim(s) 42-48, drawn to methods of selecting targeted vectors.
Group IV, claim 57, drawn to polypeptides which bind CD4.

The inventions listed as Groups I-IV do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: As evidenced by Somia, targeted vectors were known in the art prior to Applicant's invention and, therefore, the claimed inventions lack unity of invention. Further, the vector libraries of Group II contain multiple targeted vectors capable of having multiple specificities. Therefore, the inventions of Groups I-IV do not share a special technical feature within the meaning of PCT Rule 13.1 so as to form a single general inventive concept.